TITLE: Studies on the Storage Stability and Biological Variation of Glutathione Peroxidase in Blood Cells and Plasma

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SUMMARY Glutathione peroxidase (GSH-Px) is a selenium (Se)-dependent enzyme. GSH-Px can be used as a tool to assess the Se status of the human body especially in those undergoing total parenteral nutrition or living in Se-deficient areas. In this study, the storage stability and biological variation of GSH-Px activity in plasma and red cells were investigated. The method for GSH-Px assay was based on the coupled assay of Paglia and Valentine (9). Samples of plasma, red cells and haemolysate in different anticoagulants were stored at different temperatures. It was found that the enzyme was most stable at -20°C and could not be kept at room temperature without appreciable change in activity. GSH-Px was more stable in red cells than in haemolysates. Citrate was found to be better than EDTA and heparin retaining GSH-Px activity. Serial studies of GSH-Px activity in plasma and red cells of 14 healthy subjects provided data on biological variation. Samples were drawn on five occasions over a period of ten weeks. Different components of variation (analytical inter- and

intraindividual variations) were calculated. It was found that the ratio of the average within-subject variance to the variance among subjects was less than 0.6, this figure indicating that the conventional reference limits of GSH-Px were of limited use and that each individual needed his or her own reference interval.

INTRODUCTION

Selenium (Se) is firmly established as an essential nutrient for humans. Important food sources of Se are seafoods, liver, kidney and muscle meats; fruits and vegetables are generally low in Se. Grain products vary in their Se content, depending on geographical location. Low blood levels of the element have been reported in individuals from several countries where the soils are low in Se, e.g. New Zealand, parts of Finland and China. Se deficiency has been suggested as a cause of several pathological conditions such as cardiomyopathy, muscle pain, muscle weakness and susceptibility to variety of drugs and chemicals (1). One of the most well-known problems is the cardiomyopathy known as Keshan disease. The mechanism by which Se deficiency allows the development of Keshan disease is unknown. Se functions as an antioxidant in vivo and one hypothesis is that it

prevents oxidant injury of the myocardium. Such damage may be due to depletion of oxidant defences alone or could be associated with oxidant stresses caused by infections or drugs (2). It has also been shown in animals that adequate Se levels protect liver membranes against diquat and paraquat poisoning (3). Animals deficient in Se had increased susceptibility to liver necrosis. Apart from the geographical factors, it has been noticed that Se deficiency is associated with intravenous hyperalimentation, because Se supplementation of parenteral nutrition fluids has not been common practice until recently. In 1979, Van Rij et al found whole blood Se to be lower in patients on parenteral nutrition than in their normal population. The deficiency and physiologic consequences of this deficiency can be corrected with the administration of intravenous Se in the form of selenous acid. (4)

Measurement of Se in blood can be used to monitor the Se status of patients receiving total parenteral nutrition and of residents of Se deficient areas. Historically, determination of Se in biological matrices is difficult and tedious. The concentrations present are usually below those that can be determined with simple flame atomic absorption instruments, making it necessary to extract the metal and thus concentrate it.

Currently, the most commonly used method is graphite furnace atomic absorption spectrometry with Zeeman background correction which frees the Se determination from several interferences such as those iron and phosphorus (5). It would be advantageous if a simpler method could be used in the assessment of Se status.

In 1973, Se deficiency was found to be associated with glutathione peroxidase (GSH-Px) (EC 1.11.1.9) deficiency. GSH-Px was discovered to contain Se in the form of selenocysteine. The active site of GSH-Px contains one selenocysteine residue inserted in each of the enzyme's four polypeptide chains. GSH-Px plays an important role in the detoxification of hydrogen peroxide, organic hydroperoxides and lipid peroxides by oxidation of reduced glutathione, and thereby protects against oxidative damage. A highly significant correlation between body Se concentration and GSH-Px activity in whole blood, plasma and erythrocyte has been reported. This enzyme may be used as a tool to assess the Se status of the human body especially those receiving total parenteral nutrition or those from Se deficient areas. Although GSH-Px is Se dependent, there is a level of Se above which further increases in its availability cause no further elevation of enzyme activity : i.e. 'saturation' occurs (6). In general,

studies of normal, healthy subjects with what is assumed to be an adequate intake of Se have failed to find a positive correlation between the metal and the enzyme in blood or plasma (5). There have been many studies on the Se and GSH-Px levels in plasma and blood cells of patients receiving parenteral nutrition or living in Se deficient areas. It has been commonly found that Se and GSH-Px levels are significantly lower in those people. When the replacement of Se was commenced, there was a rapid increase in plasma GSH-Px. Within 6-12 hours after Se therapy was instituted, there was a demonstrable increase in plasma enzyme activity with no apparent lag. The recovery rates of red cell and platelet GSH-Px activity were similar to their rates of production (7).

In this project, no further investigations in the above aspects were carried out. What I was interested in were the storage stability and biological variation of GSH-Px in blood cells and plasma. The technical aspects of GSH-Px assay will also be discussed. Since no systematic discussions on the storage stability and biological variation of GSH-Px could be found in the literature, it was decided to obtain some data in the present study. Blood samples collected in different anticoagulants (citrate, EDTA and heparin) and stored at

different temperatures were used in the storage stability study. 14 healthy adults were invited to donate blood over a period of 10 weeks, and their data were used in the analysis of the biological variation of GSH-Px.

METHODS

Subjects 14 young and healthy (10 males and 4 females) laboratory staff were asked to give blood samples for the project. They were aged 21-31 years. Blood was sampled at 0, 1, 2, 5 and 10 weeks. During the 10-week period, the subjects were on a normal diet and 10ml of venous blood was taken from each at 9-10 am in the morning on each occasion. They were not requested to fast and blood was taken after being seated for 5 minutes.

Sample treatment The 10ml blood sample of each individual was divided into three different anticoagulant bottles : 8ml in citrate (0.9ml 0.106mol/L citrate:8ml blood), 1ml in EDTA (1mg EDTA/ml of blood), and 1ml in heparin (12-15 U/ml of blood). EDTA and heparinized blood samples were centrifuged at 1500g for 5 minutes to separate red cells from plasma. The former

were washed three times with isotonic saline, and the plasma kept for study. Since platelets can only be extracted from citrated blood, the blood sample in citrate was centrifuged at low speed (200g for 10 minutes) to obtain platelet-rich-plasma (PRP). The citrate red cells were also washed three times with isotonic saline. Two additional low speed centrifugations of PRP were incorporated to get rid of any red cells. The platelets could then be obtained by high speed centrifugation (3000g for 15 minutes). The platelet pellet was washed three times with isotonic magnesium chloride (110 mmol/L). The citrate plasma was also kept for further studies. The plasma GSH-Px activity in different anticoagulants could be directly determined without any special treatment. However, red cell and platelet GSH-Px activities could only be assayed after further treatment. 1 part of red cells was lysed with 3 parts of 0.02% saponin and the haemolysate then diluted with 10 parts of deionized water. After thorough mixing, the 1 in 40 haemolysate was centrifuged at 1500g for 15 minutes to remove debris. The clear supernatant was suitable for assay. Sonication has been used to disrupt platelets (8). Others have obtained platelet lysates by resuspending fresh centrifuged platelet pellets in saturated digitonin medium at $4^{\circ}C$ for 1 hour and using the

supernatants to test platelet GSH-Px activity (6). In this project, saponin was investigated as a lysing agent for platelets. 0.1% saponin (0.5 ml) was added to each platelet pellet and stood overnight at room temperature, and the supernatant used for GSH-Px assay. The reason for using saponin as a lysing agent was that it has been used satisfactorily in the preparation of platelet lysates for ATPase assay in a project in this department (Robertshaw, AM. personal communication). As a result, saponin was evaluated to see whether it could also be used in the platelet GSH-Px assay.

Protocol In the storage study, samples of plasma, washed red cells and platelet in different anticoagulants were obtained from the pooled specimens of the first blood taking of 14 volunteers. Moreover, haemolysates of red cells were also prepared to compare GSH-Px stability in red cell and haemolysate. The GSH-Px activities in these various types of specimen were tested on alternate days during the first two weeks and then once a week for another 3 weeks.

In the biological variation study, blood samples obtained on each occasion were processed and GSH-Px activities in various blood components were assayed within 12 hours.

The enzyme activity in plasma was expressed as international units (1 umol of substrate coverted / minute) per litre (U/L), in red cells as units per gram of haemoglobin (U/gHb) and in platelets as units per gram of protein (U/g).

Equipment, reagents and assays In this project, the analytical method of GSH-Px assay was based on that described by McMaster et al (5) with some modifications. The method used by McMaster et al was based on the coupled assay of Paglia and Valentine (9).

For the estimation of enzyme activity in plasma, red cells and platelets, the Cobas Bio centrifugal analyzer (F. Hoffmann-La Roche & Co. Limited, Balse, Switzerland). GSH-Px catalyzes the oxidation of reduced glutathione (GSH) by hydrogen peroxide (H_2O_2). The substrate is maintained at a constant concentration by the addition of glutathione reductase (GSSG-R) and reduced nicotinamide adenine dinucleotide phosphate (NADPH). The decrease in absorbance of the reaction mixture at 340nm is a measure of oxidized glutathione (GSSG) production. Therefore, higher GSH-Px activity gives greater decrease in absorbance.

$$2GSH+H_2O_2 \xrightarrow{GSH\text{-}Px} GSSG+2H_2O$$

$$+$$

$$H^+ + NADPH - \xrightarrow{GSSG\text{-}R} 2GSH+NADP^+$$

NADPH tetrasodium salt type 1, glutathione (reduced form) and glutathione reductase (EC 1.6.4.2) (type IV) were purchased from Sigma Chemical Co. St. Louis, U.S.A.

A litre of the stock phosphate buffer contained 10.2 g of KH_2PO_4, 30ml of 0.1 mol/L Na_2EDTA, and 0.4 g of NaN_3. The pH was brought to 7.0 with a 1 mol/L solution of NaOH. The concentration of NaN_3 was 1000 times higher than in the method of McMaster et al. The reason for using this concentration of NaN_3 will be discussed later.

The enzymatic reaction needed two reagents : the principal reagent and start reagent. The principal reagent contained 8.67 ml of stock phosphate buffer, 3.5 mg of NADPH, 10 mg of GSH, 6 U of GSSG-R, and 0.5 ml of Drabkin's reagent. The start reagent contained 34 ul of 7.2 mol/L H_2O_2 in 100 ml of water. Since stock H_2O_2 is not very stable even at 4^oC, its concentration should be confirmed monthly by indirect titration. Because of the problem of stability, both reagents were prepared immediately before use and kept at 4^oC in an ice bath. The test program for GSH-Px is shown in (Table 1). A 100 uL sample was transferred to an analyser cup, and the enzyme activity assayed automatically by the

Table 1 Cobas Bio test program for GSH-Px

1. Units	U/L
2. Calculation factor	4019
3. Standard 1 conc.	0
4. Standard 2 conc.	0
5. Standard 3 conc.	0
6. Limit	0.3300
7. Temperature (Deg. C)	37
8. Type of analysis	3
9. Wavelength (nm)	340
10. Sample volume (uL)	10
11. Diluent volume (uL)	20
12. Reagent volume (uL)	90
13. Incubation time (sec)	60
14. Start reagent volume (uL)	10
15. Time of first reading (sec)	60.0
16. Time interval (sec)	10
17. Number of readings	7
18. Blanking mode	1
19. Printout mode	2

computer-controlled instrument. With the test program, the sample, diluent, and principal reagent were pipetted simultaneously into the analyser rotor and mixed. A blank, consisting of reagent and diluent, was automatically included. Start reagent (H_2O_2) was added to the cuvettes after incubation of mixture for 60 seconds at 37°C. After acceleration and mixing, the absorbance was read at the pre-programmed time intervals. All calculations were based on the change in absorbance between readings 1 and 7. The rate of change of absorbance for each sample was calculated in absorbance units per minute and corrected for the corresponding measurement in the blank. The net rate of change of absorbance ($\Delta a/t$) was a measure of the enzyme activity. The specific activity of GSH-Px in U/L of sample is derived as follows :

$$\text{GSH-Px (U/L)} = F. \Delta a/t$$

The calibration factor F was derived from physical coefficients, the stoichiometry of the reactions, the geometry of the cuvette, and the volume of sample introduced into the cuvette. In this protocol, F was equal to 4019 (5).

Enzyme activity in red cells was expressed as

international units per gram of haemoglobin (U/gHb). Haemoglobin concentration in each haemolysate was determined using the Cobas Bio. The reagent and haemoglobin standard were supplied by Sigma Chemical Co. St. Louis, U.S.A. A vial of Drabkin's reagent (Catalogue No. 525-2) was reconstituted in 400 ml water, giving a concentration 2.5 times that of the commonly used reagent. 4 ml of concentrated Drabkin's reagent was added to a vial of haemoglobin standard (Catalogue No. 525-18). This standard was equivalent to 9 gHb/L. The linearity of this reaction was from 3-30 gHb/L. Table 2 showed the test program for haemoglobin.

In the platelet assay, the enzyme activity was expressed as international units per gram of protein (U/g). The protein concentration of the platelet lysate was determined with the Bio-Rad procedure in the Cobas Bio. This assay is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Beer's Law may be applied for accurate quantitation of the protein by selecting an appropriate ratio of dye volume to sample concentration. The Coomassie Brilliant Blue G-250 was obtained from

Table 2 Cobas Bio test program for haemoglobin

1. Units	g/L
2. Calculation factor	0
3. Standard 1 conc.	9.00
4. Standard 2 conc.	9.00
5. Standard 3 conc.	0
6. Limit	0
7. Temperature (Deg. C)	25.0
8. Type of analysis	1
9. Wavelength (nm)	540
10. Sample volume (uL)	10
11. Diluent volume (uL)	50
12. Reagent volume (uL)	90
13. Incubation time (sec)	10
14. Start reagent volume (uL)	0
15. Time of first reading (sec)	0.5
16. Time interval (sec)	180
17. Number of readings	10
18. Blanking mode	1
19. Printout mode	1

Bio-Rad Chemical Division, California, U.S.A. One part of stock reagent was diluted with four parts of deionised water. The standards were prepared by diluting the (CSF) calibrator 4 of Astra Systems (Beckman Instruments Inc. U.S.A.) with 0.1% saponin. The assay was linear over the range 10-220 mg/L. The test program for low concentration protein in platelet lysate is shown in (Table 3).

RESULTS

Technical aspects The method for the GSH-Px assay of this project was adopted from the paper of McMaster et al (1990) (5). Reagents were prepared according to their description. It was found that there was no problem in the reaction of plasma and serum GSH-Px which gave enzyme activities of similiar magnitude to other reports. However, the enzyme activity in haemolysate could not be determined by the centrifugal analyzer and the rate of decrease in absorbance was even smaller than the blank. The reaction profiles of the enzymatic reaction were then investigated. The absorbance readings of reaction from time 0 to 190 seconds were recalled and plotted on graphs. It was obvious that the plasma gave a perfectly linear reaction (Fig. 1), but

Table 3 Cobas Bio test program for protein

1. Units	mg/L
2. Calculation factor	0
3. Standard 1 conc.	0.0001
4. Standard 2 conc.	52.8
5. Standard 3 conc.	211.2
6. Limit	0
7. Temperature (Deg. C)	25.0
8. Type of analysis	1
9. Wavelength (nm)	595
10. Sample volume (uL)	4
11. Diluent volume (uL)	20
12. Reagent volume (uL)	200
13. Incubation time (sec)	10
14. Start reagent volume (uL)	0
15. Time of first reading (sec)	0.5
16. Time interval (sec)	120
17. Number of readings	2
18. Blanking mode	1
19. Printout mode	1

Reaction profile of GSH-Px in plasma

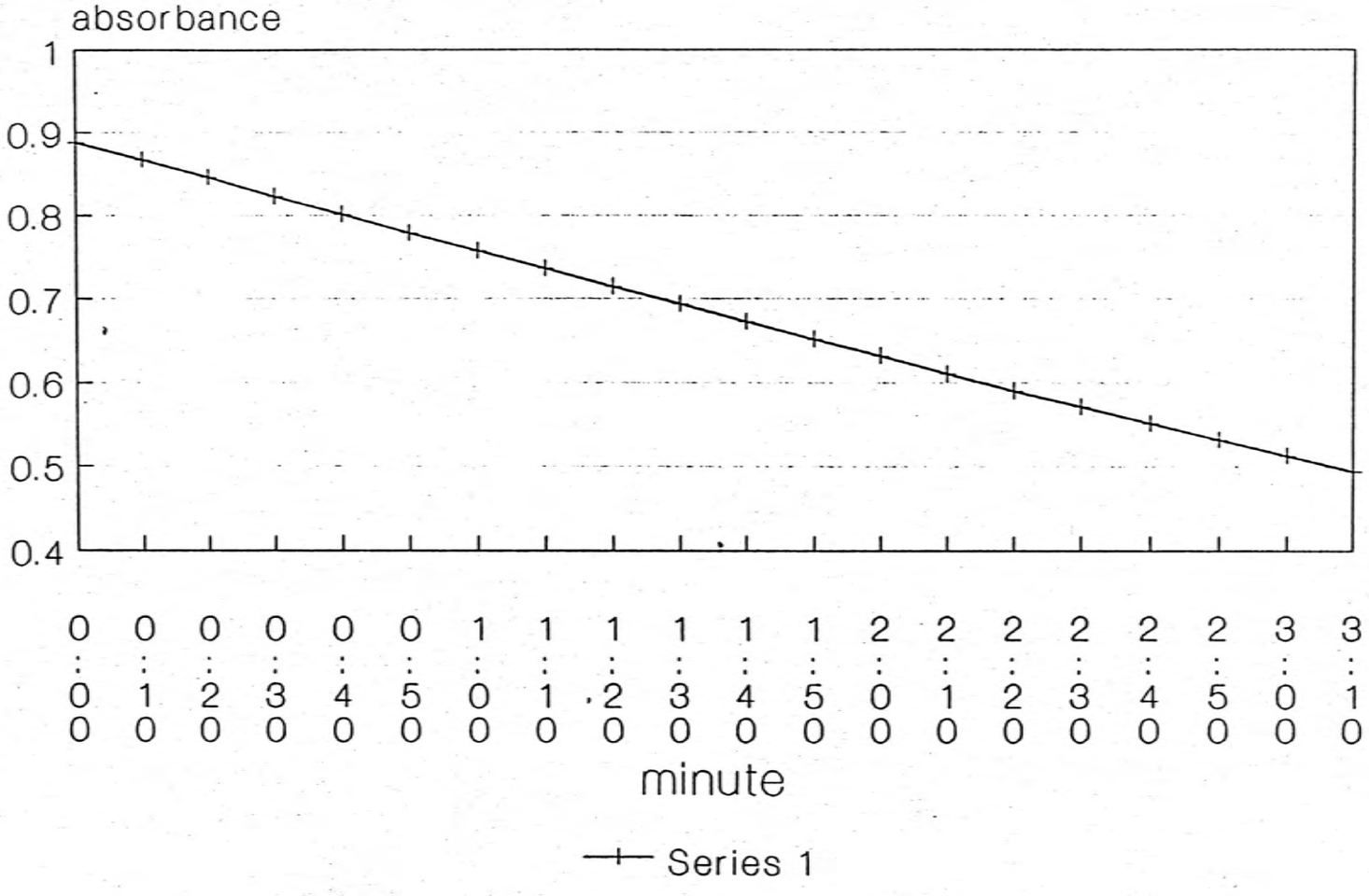


Fig.1

the reaction profile of haemolysate was non-linear, the absorbance only decreased at the very beginning of reaction and then flattened out quickly (Fig. 2). The first reason suspected for non-linearity in the haemolysate enzyme assay was the depletion of substrate. Therefore, a higher substrate ([NADPH] x 2) concentration was tried, and the profile was plotted (Fig. 3). It was clear that there was no improvement in linearity and insufficiency of substrate was unlikely. After reading the paper of Paglia and Valentine (9) which gave detailed accounts of the quantitative and qualitative characterization of erythrocyte GSH-Px, further investigations were made. Their method used double-strength Drabkin's reagent in the reaction mixture, so this was assessed. Unfortunately, the reaction was still not linear (Fig. 4). Increasing cyanide concentration (5 times, and 10 times) was also studied. It was disappointing that a linear reaction could not be observed (Fig. 5). Although those studies did not reveal a cause for the non-linearity, another was made by increasing azide concentration. Compared with the reagent compositions quoted in the paper of Pleban P A et al (10), it was found that all the concentrations of reagents were similar in magnitude except sodium azide. The sodium azide concentration was a thousand-fold lower in the original method. With this

Reaction profile of GSH-Px in hemolysate

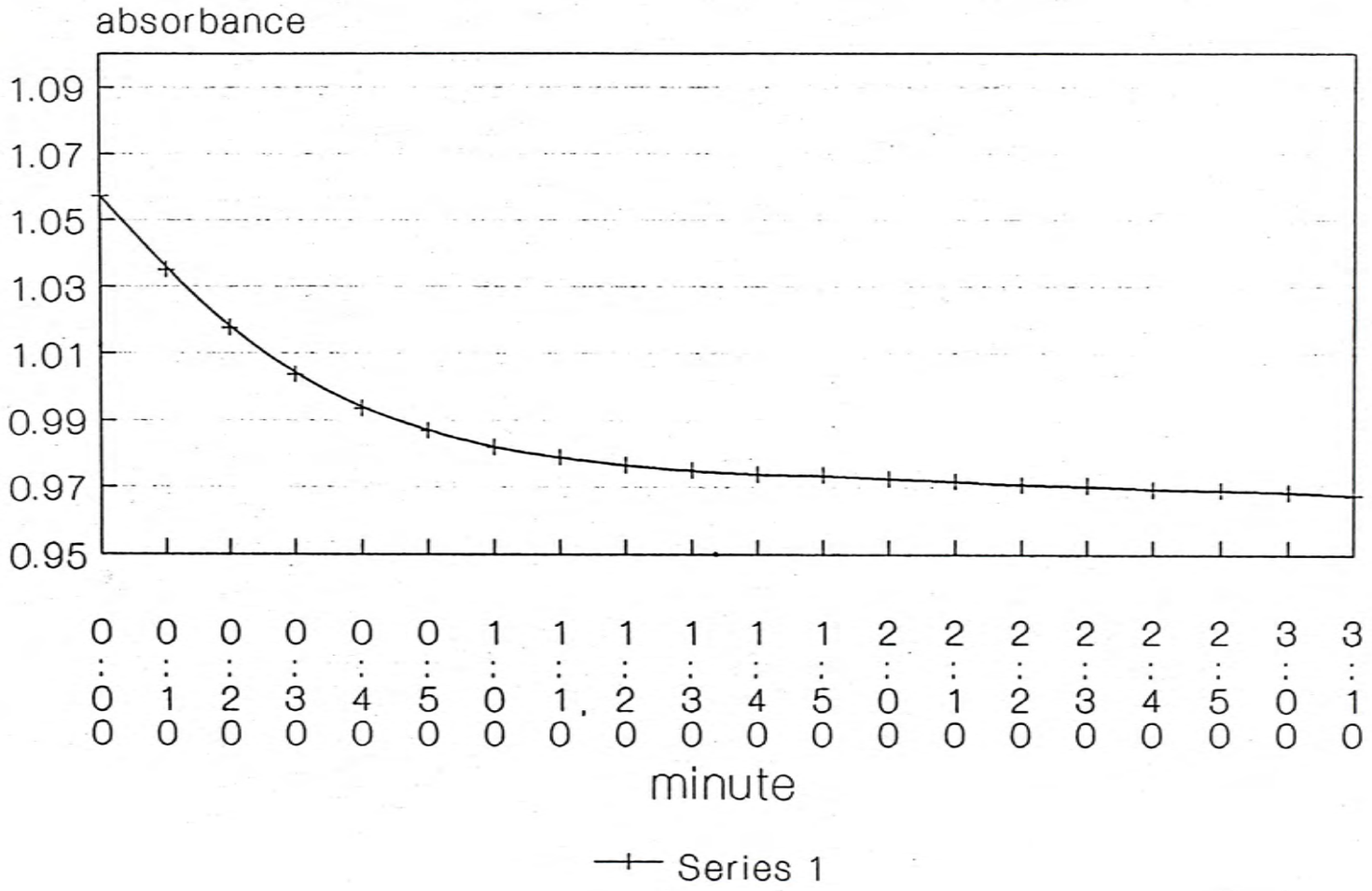


Fig.2

Reaction profile of GSH-Px in hemolysate

Double substrate (NADPH) concentration

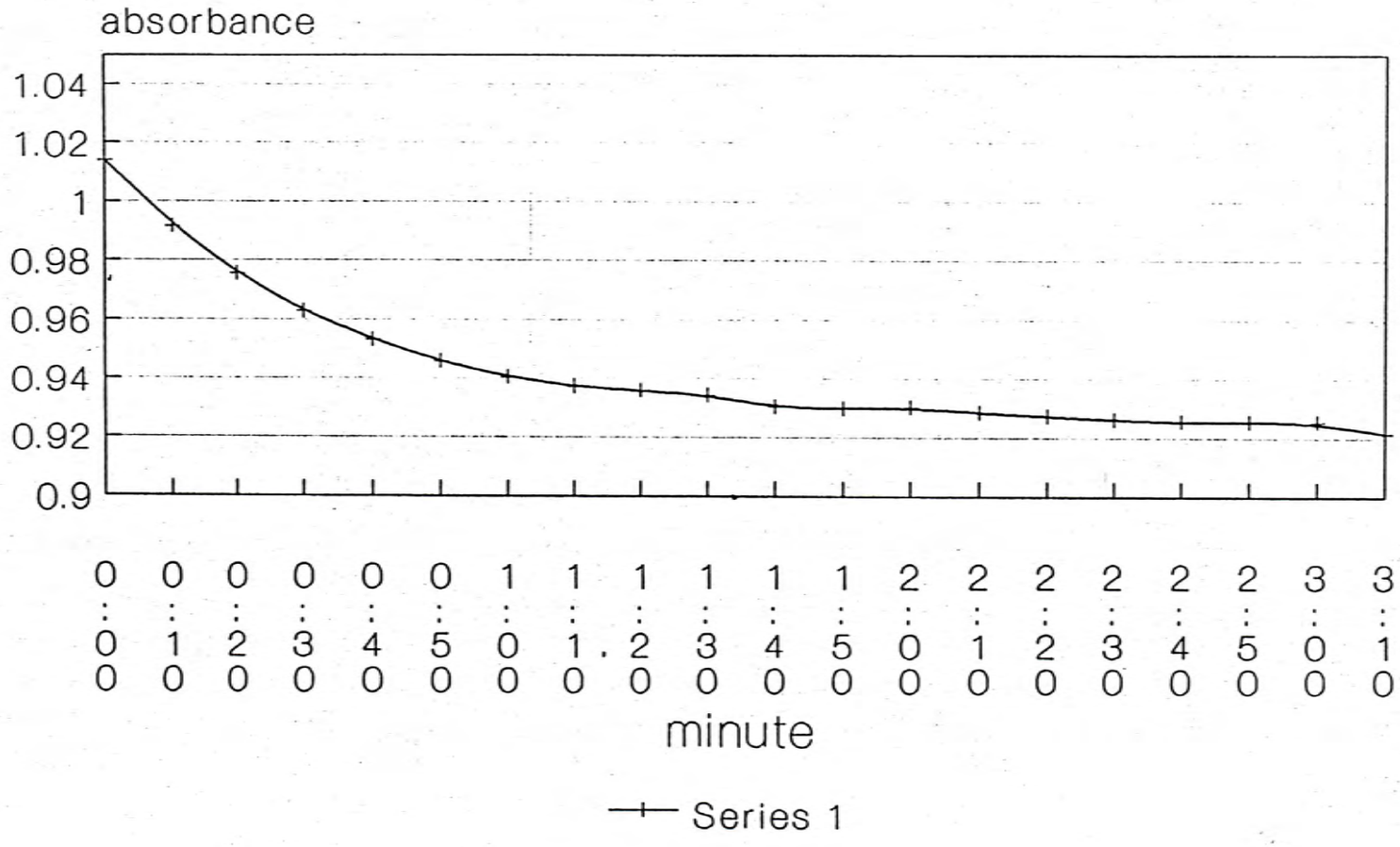


Fig.3

Reaction profile of GSH-Px in hemolysate

Double strength Drabkin's reagent

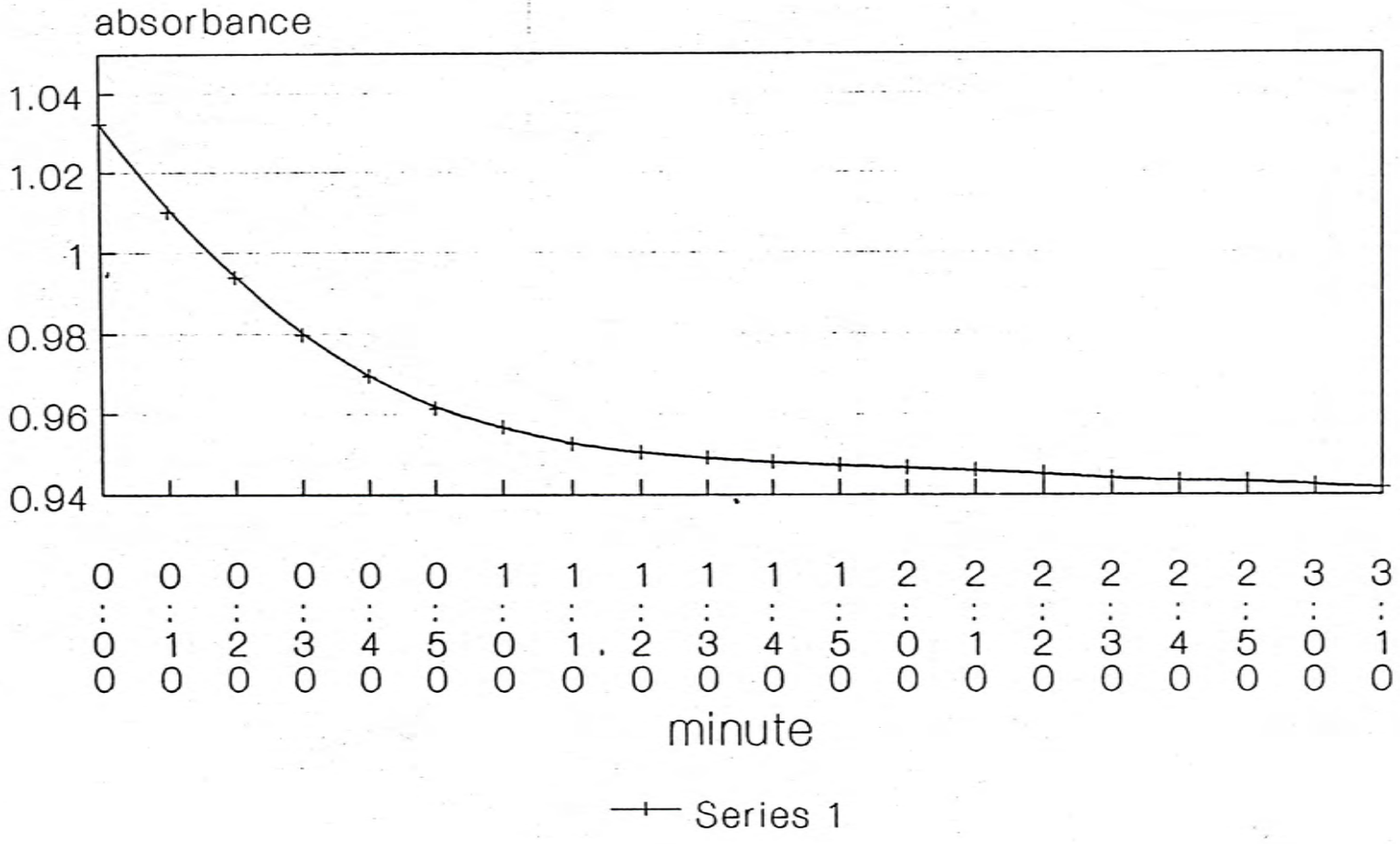


Fig.4

Reaction profile of GSH-Px in hemolysate

Increase cyanide concentration (X5, X10)

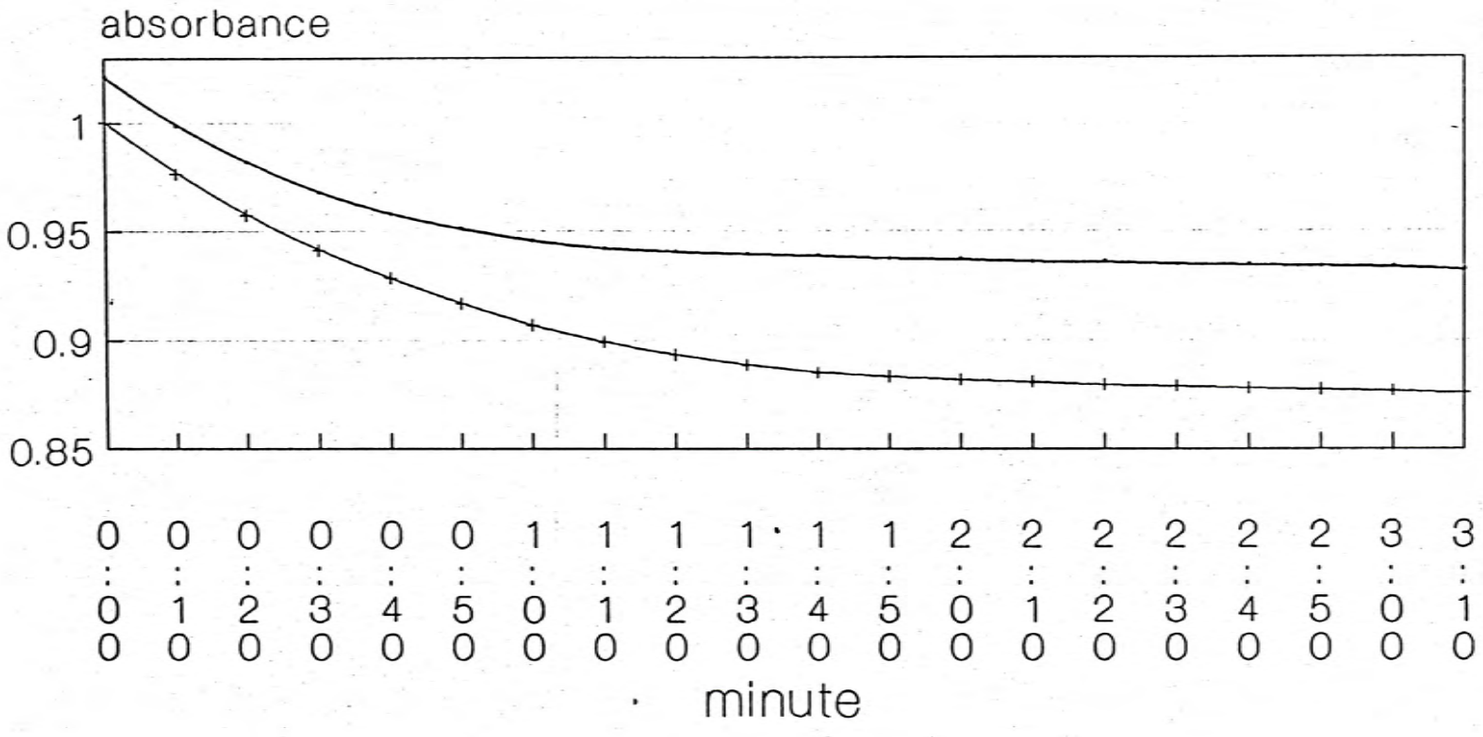


Fig.5

finding, buffers of 100 times (0.62 mmol NaN_3/L phosphate buffer) and 1000 times (6.2 mmol NaN_3/L phosphate buffer) azide were prepared and evaluated. A great improvement was now observed, both buffers gave nearly linear reaction profiles (Fig. 6). The new buffers were then evaluated for the assay of GSH-Px in haemolysates. It was found that buffer of 0.62 mmol/L NaN_3 gave results with noise while 6.20 mmol/L NaN_3 buffer had perfect results without any noise. The buffer containing 6.20 mmol/L NaN_3 was therefore chosen for the determination of plasma and red cell GSH-Px activities in this project. From the above observations, it was concluded that the reason of non-linearity in haemolysate enzyme reaction was due to insufficiency of azide in buffer. It is assumed there was a misprinting of sodium azide concentration in the paper of McMaster et al (5) (i.e. 0.4 mg/L instead of 0.4 g/L).

The platelet lysate could not be successfully prepared in this project, therefore no data could be provided in the stability and biological variation studies. Saponin was evaluated as a lysing agent for the platelets where other researchers have used sonication or digitonin (6, 7, 8). Different concentrations of saponin (0.02%, 0.05%, and 0.1%),

Reaction profile of GSH-Px in hemolysate

Increase sodium azide concentration (100x, 1000x)

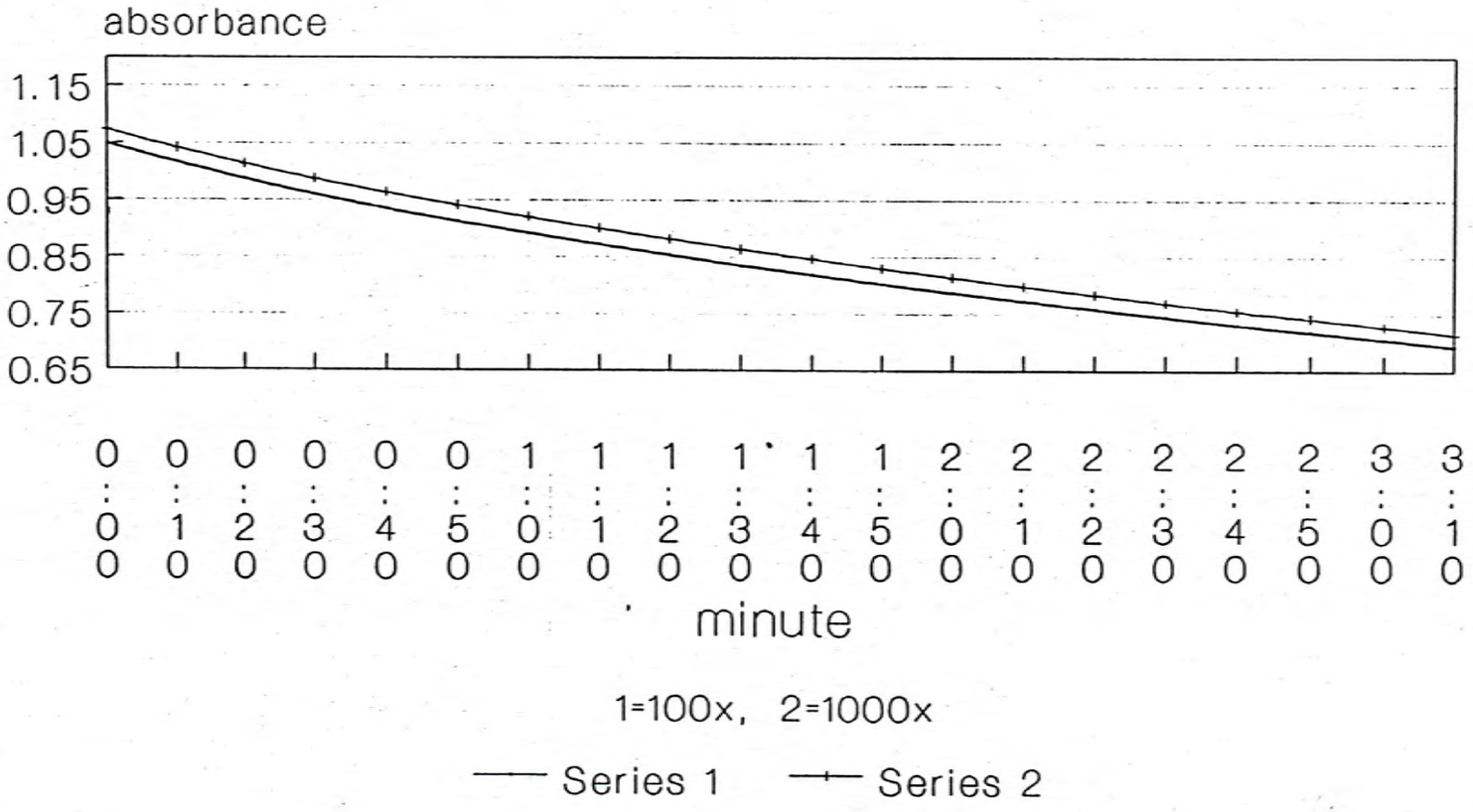


Fig.6

different methods of resuspension of platelet pellet (gently rotary mixing and vortex mixing) and different durations of lysing period (20 minutes, 4, 8, 12 or even 16 hours) were evaluated, but reliable and reproducible results could not be obtained. It may be that saponin is not a suitable lysing agent for platelet. Due to the constraint of time and availability of chemicals, the platelet assay could not be optimized in time for inclusion in the project.

Assay characteristics The centrifugal analyzer used for GSH-Px assay was found to be satisfactory. The within-run and between-run precisions of enzyme activities in plasma and red cells were acceptable, and similar to those in other reports (5, 10). For the within-run precision, 25 aliquots of a plasma sample and 20 or 13 haemolysates of a washed red cells specimen were prepared, and then analyzed within the same batch. The data of between-run precision were obtained from eleven separate runs of the same blood sample. The results of precision studies are shown in (Table 4 and 5). Obviously, when the coefficients of variance were compared, the analytical imprecision of red cell enzyme activity was greater than plasma.

Stability In the literature review, no systematic

Table 4 Within-run precision of the assay for GSH-Px in plasma and red cells

Sample*	n	mean±SD	CV(%)
CP (U/L)	25	358±3.8	1.1
EP (U/L)	25	433±4.7	1.1
HP (U/L)	25	458±6.7	1.5
CR (U/gHb)	20	37.6±2.11	5.6
ER (U/gHb)	20	39.7±2.60	6.6
HR (U/gHb)	13	36.6±1.30	3.6

* CP = citrate plasma, EP = EDTA plasma,
HP = heparin plasma, CR = citrate red cells,
ER = EDTA red cells, HP = heparin red cells

Table 5 Between-run precision of the assay for GSH-Px in plasma and red cells

Sample*	no. of runs	mean±SD	CV(%)
CP (U/L)	11	367±10.7	2.9
EP (U/L)	11	429±16.4	3.8
HP (U/L)	11	439±11.0	2.5
CR (U/gHb)	11	38.6±3.72	9.7
ER (U/gHb)	11	39.7±3.61	9.6
HR (U/gHb)	11	42.9±3.26	7.6

* CP = citrate plasma, EP = EDTA plasma,
HP = heparin plasma, CR = citrate red cells,
ER = EDTA red cells, HP = heparin red cells

discussion on the storage stability of GSH-Px was found. In this project, blood samples were collected in three different anticoagulants (citrate, EDTA and heparin), plasma, washed red cells and red cell haemolysates were freshly separated, prepared and aliquoted. These nine different types of samples were then stored at three different temperatures (room temperature, 4°C and -20°C). The enzyme stability in different types of specimen, different anticoagulants and at different temperatures could be verified in this setting. Table 6 shows the GSH-Px activities of each type of samples over a period of 5 weeks. The stabilities of GSH-Px in different types of samples are also shown graphically (Figs. 7-15). The percentage of enzyme activity remaining after 1 week, 2 weeks and 5 weeks in each type of sample is found in (Table 7).

It is clear that the enzyme (GSH-Px) deteriorated rapidly when stored at room temperature irrespective of the type of anticoagulant or specimen. Compared with red cells and haemolysates, plasma appears to lose its enzyme activity more rapidly at room temperature.

As expected, -20°C was the most desirable temperature for the storage of GSH-Px although there was a slight loss in GSH-Px activity after 5 weeks in some types of samples. A temperature of 4°C could also keep

Table 6

Storage stability of glutathione peroxidase

Day		0	2	4	6	8	10	12	14	21	26	35
Citrate	R.T.		352	280	222	209	202	137	137	76.2	59.2	69.2
plasma	4 C	358	365	355	363	362	357	304	315	271	263	184
(U/L)	-20 C		367	353	361	360	382	354	375	377	378	356
EDTA	R.T.		399	245	162	120	81.8	61.1	57.6	54.7	53.8	52.4
plasma	4 C	433	441	416	409	406	397	383	346	231	243	129
(U/L)	-20 C		438	429	439	448	448	415	435	430	399	404
Heparin	R.T.		398	243	124	81.6	66.4	59.3	53.7	41.1	45.7	45.3
plasma	4 C	458	438	395	387	342	226	307	273	189	142	105
(U/L)	-20 C		443	438	419	444	445	438	447	440	435	419
Citrate	R.T.		37.3	23.1	15.9	13.3	15.7	13.7	11.8	11.3	10.5	8.67
hemo. *	4 C	37.5	34.2	31.7	34.8	37.4	42.8	34.8	38.5	37.6	30.3	12.9
(U/g Hb)	-20 C		32.0	31.4	34.9	38.6	40.5	34.8	37.0	39.7	32.3	33.0
EDTA	R.T.		31.5	17.1	15.5	14.7	15.5	11.8	12.3	10.9	9.67	M
hemo. *	4 C	38.7	37.6	35.5	35.1	36.7	42.0	34.9	37.4	39.0	28.8	19.6
(U/g Hb)	-20 C		35.9	34.8	35.3	35.4	43.7	37.5	35.6	39.1	30.8	30.4
Heparin	R.T.		38.6	31.3	17.8	14.4	16.7	13.4	11.7	10.9	10.3	8.98
hemo. *	4 C	41.9	38.3	36.2	36.9	43.6	44.3	37.6	40.2	40.8	31.3	24.0
(U/g Hb)	-20 C		38.3	37.5	38.8	41.0	42.8	37.3	29.2	42.4	34.8	31.1
Citrate	R.T.		37.7	34.3	36.4	34.7	27.9	31.7	29.2	23.4	34.3	M
rbc *	4 C	37.5	38.5	33.3	38.5	38.5	42.6	33.6	36.4	34.9	34.0	31.5
(U/g Hb)	-20 C		39.8	36.5	40.4	44.5	46.0	38.1	37.8	34.9	35.3	33.4
EDTA	R.T.		39.9	29.1	27.0	31.7	23.9	27.1	17.8	12.8	17.7	M
rbc *	4 C	38.7	41.9	38.7	41.0	41.2	45.9	35.6	35.0	34.5	29.3	31.1
(U/g Hb)	-20 C		41.9	38.7	41.1	43.6	46.4	39.0	41.4	37.4	34.7	33.3
Heparin	R.T.		43.4	38.0	32.0	34.2	26.5	M	M	M	M	M
rbc *	4 C	41.9	44.2	41.4	43.3	46.1	49.0	36.7	45.8	38.5	34.7	34.8
(U/g Hb)	-20 C		44.4	41.7	46.3	46.5	48.1	41.9	44.2	39.2	38.8	38.8

* hemo = haemolysate, rbc = red blood cells
+ M = moulded sample

Fig. 7. **Effect of storage temperature**
(citrate plasma)

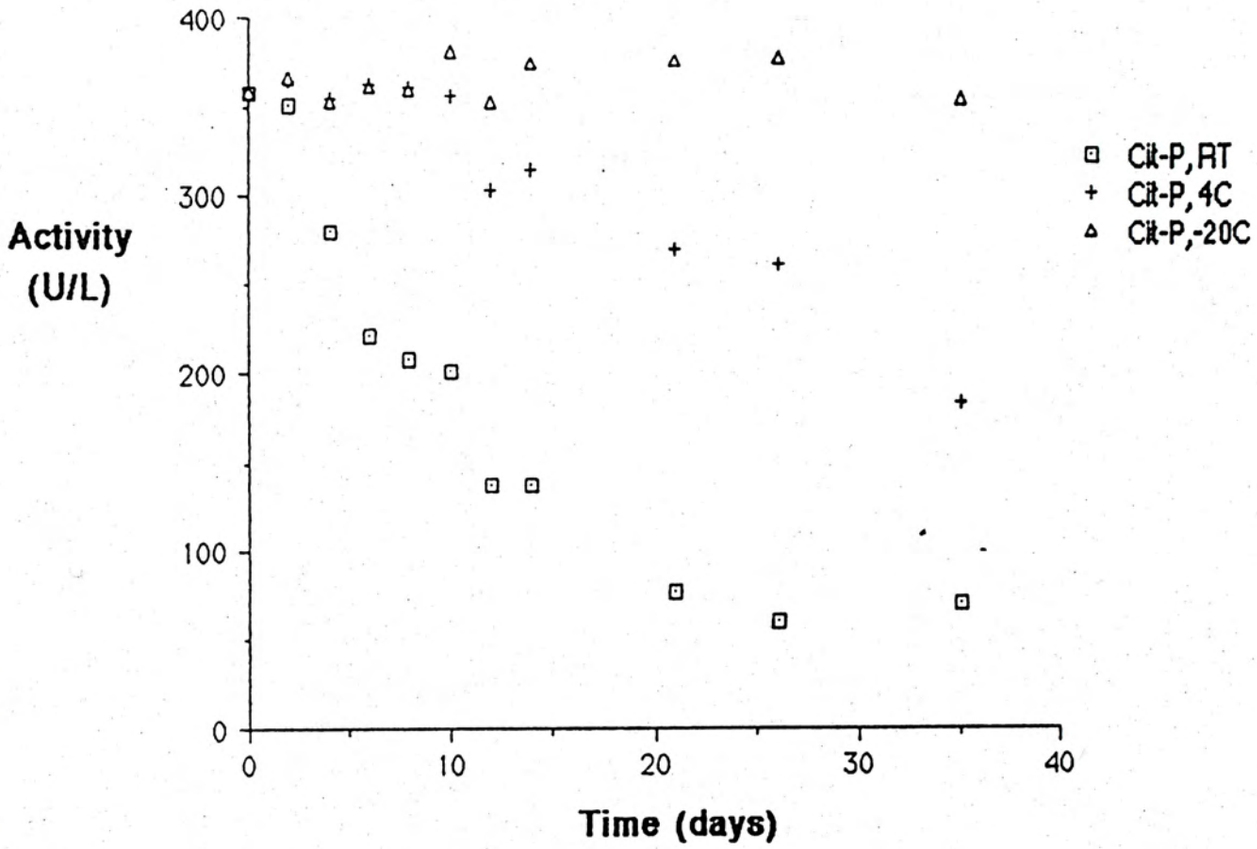


Fig. 8. Effect of storage temperature

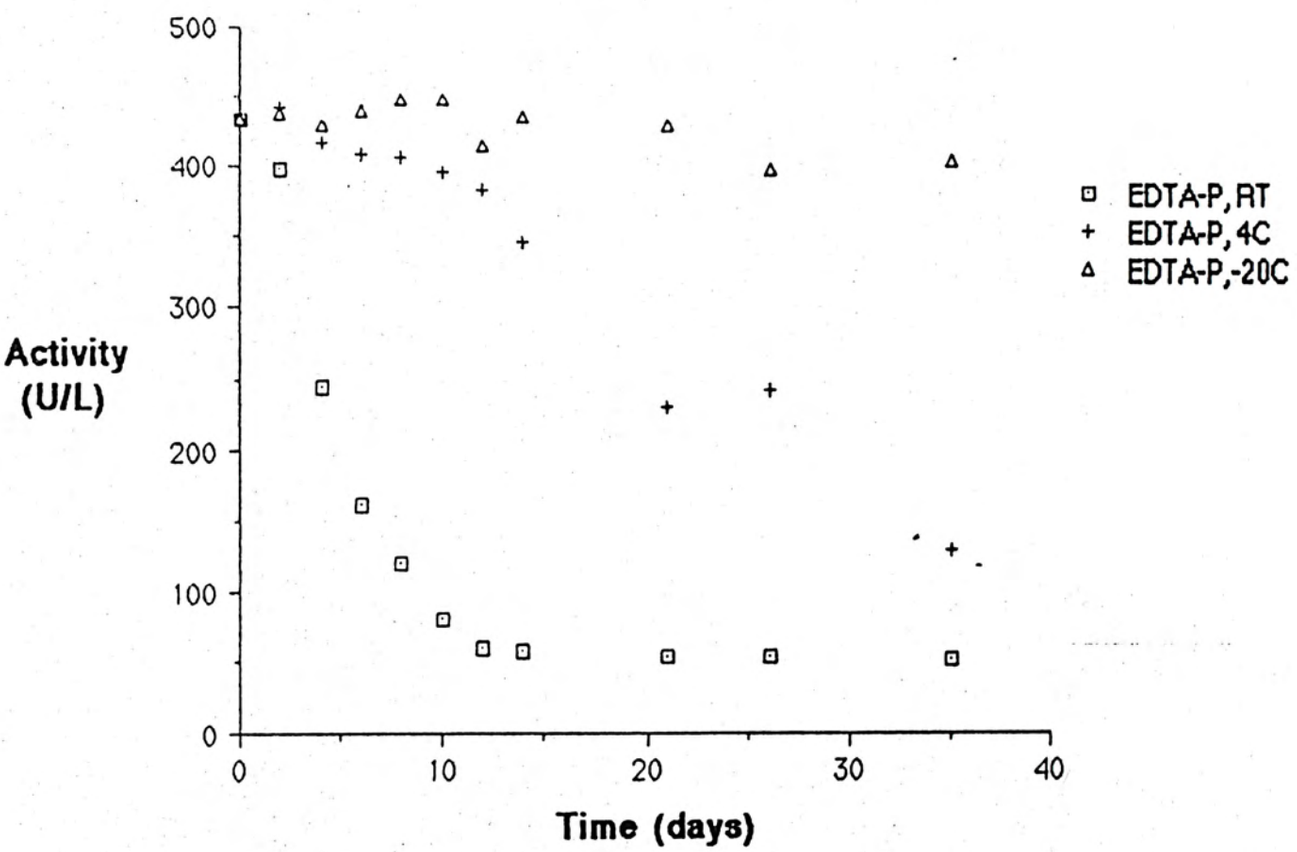


Fig. 9. **Effect of storage temperature**

(Heparin plasma)

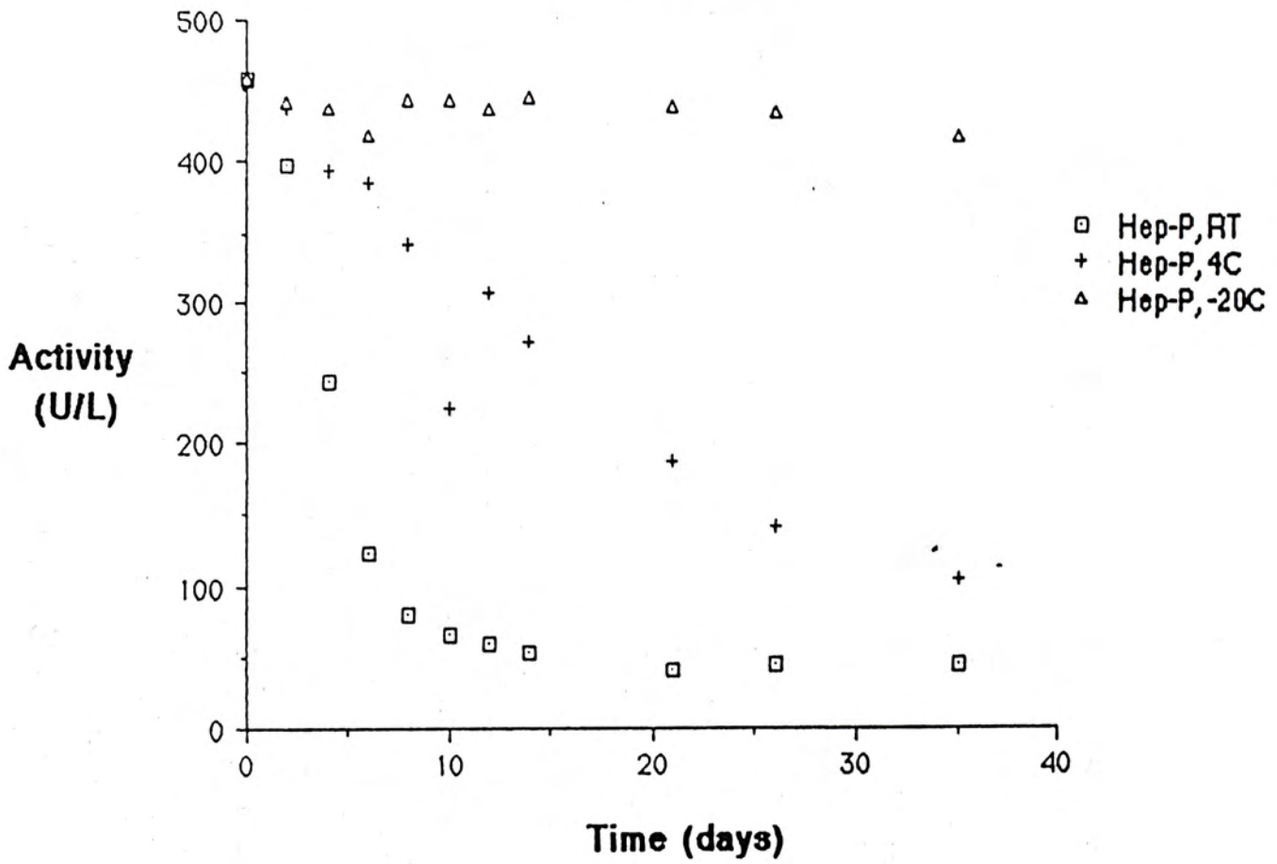


Fig. 10. **Effect of storage temperature**
(citrate haemolysate)

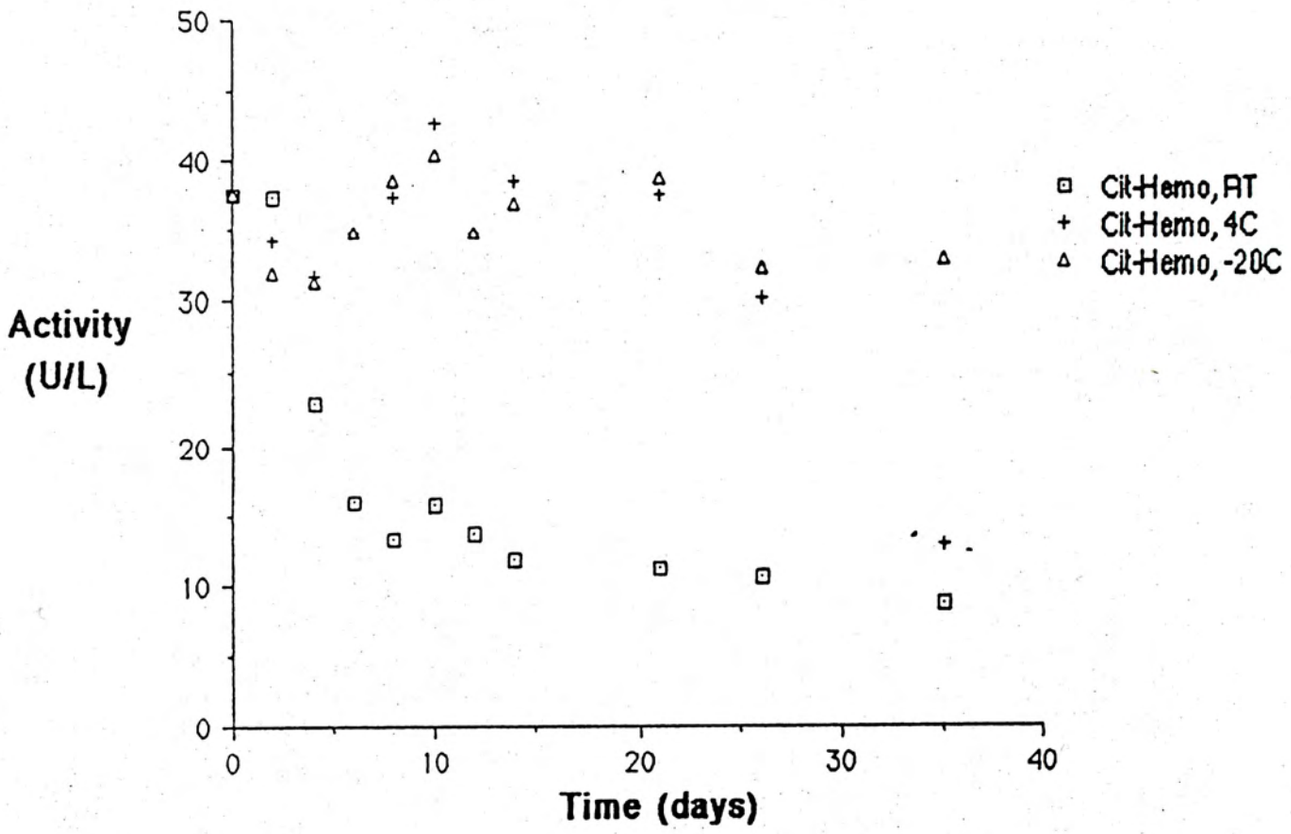


Fig. 11. **Effect of storage temperature**

(EDTA haemolysate)

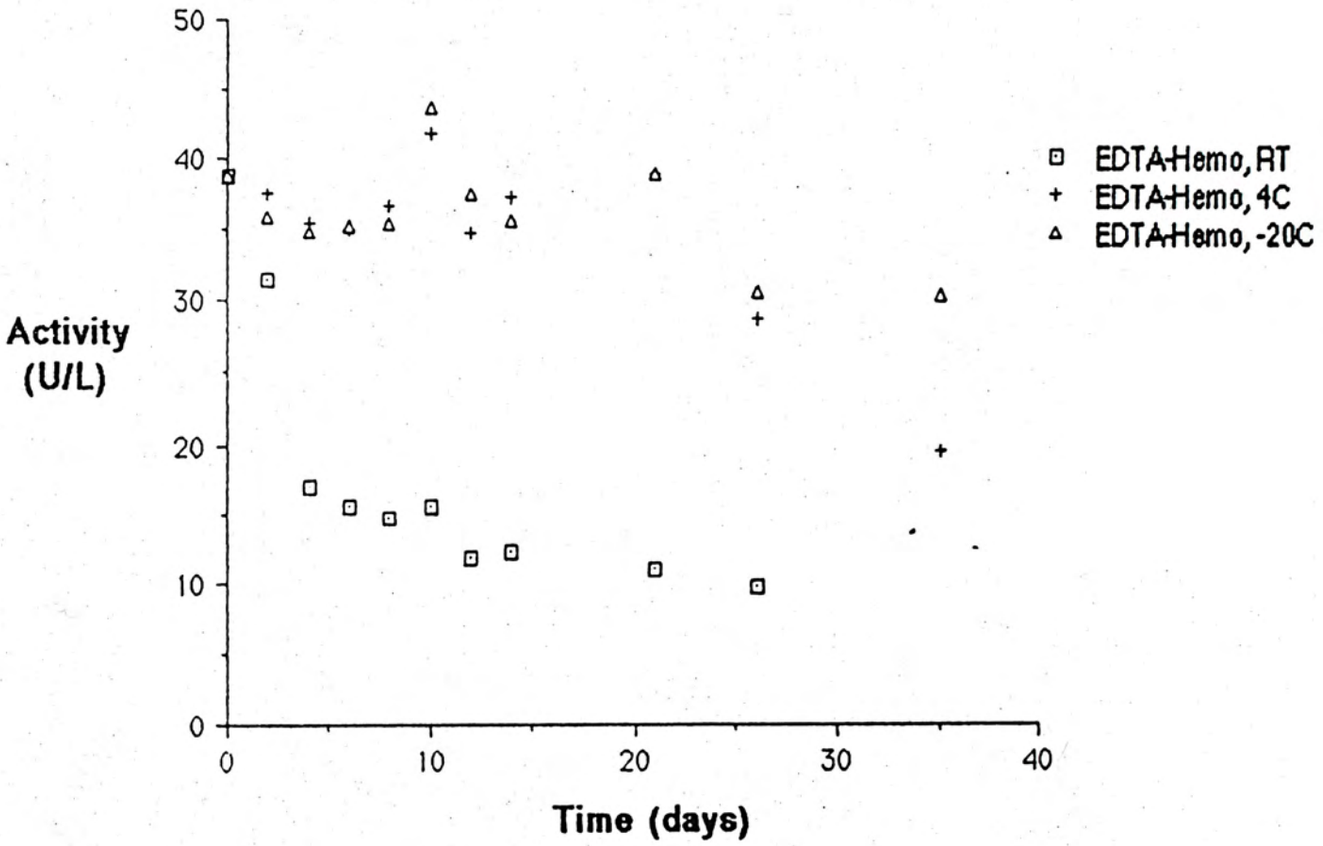


Fig. 12. **Effect of storage temperature**
(heparin haemolysate)

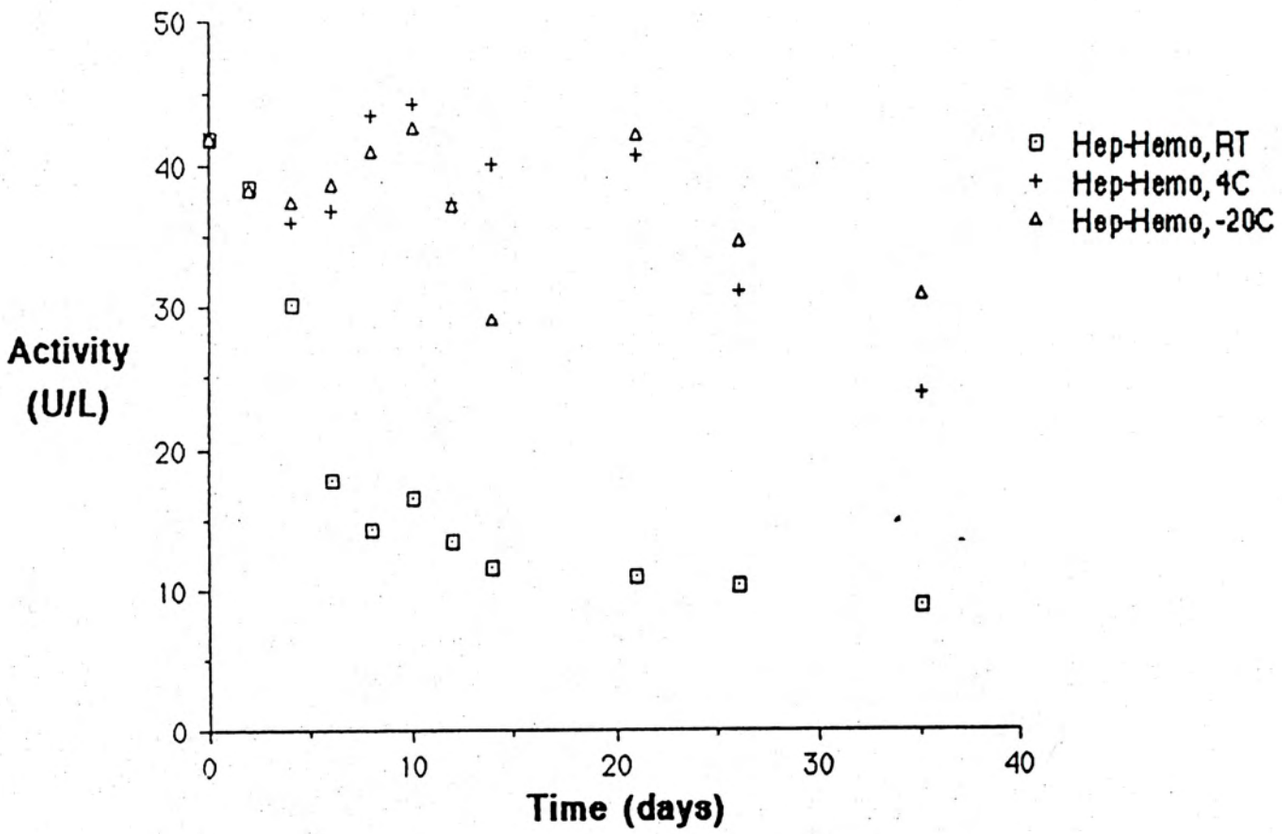


Fig. 13. **Effect of storage temperature**

(citrate red cells)

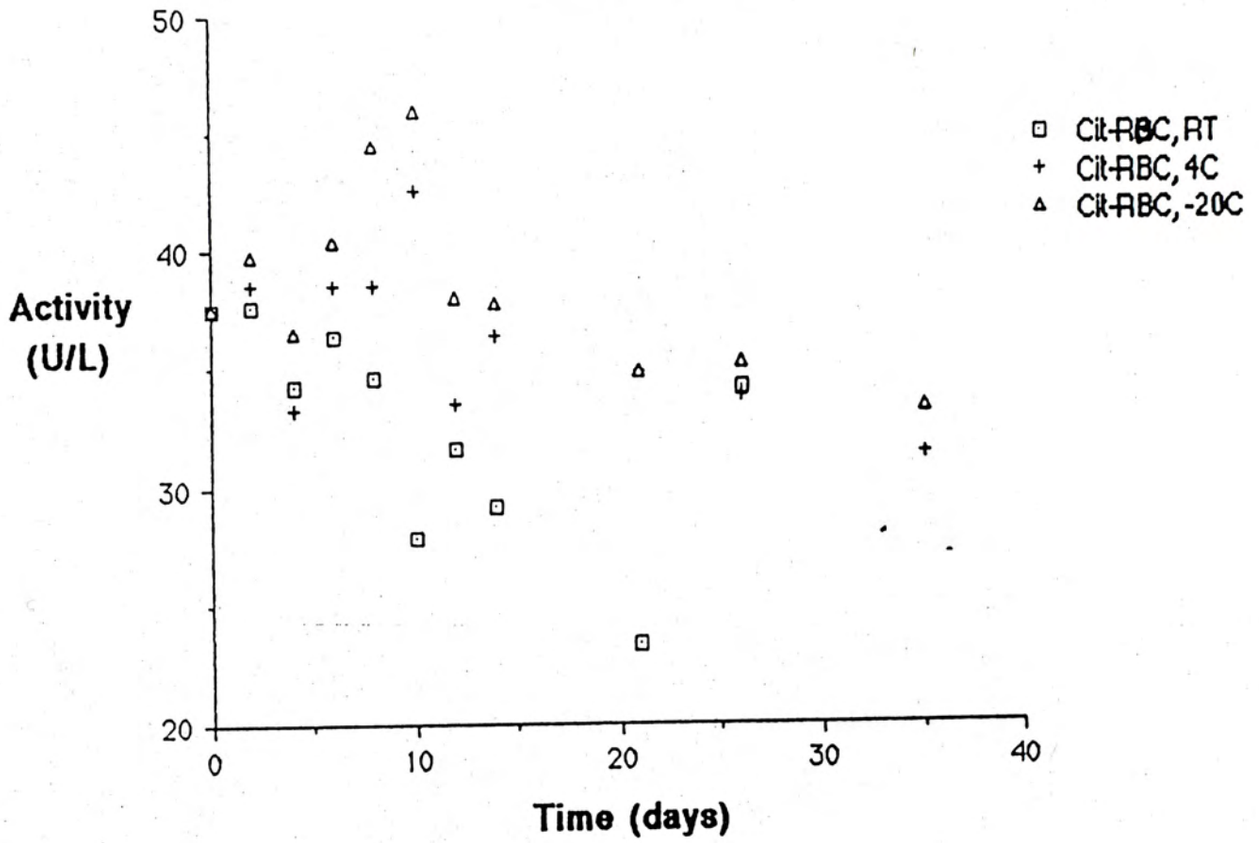


Fig. 14. Effect of storage temperature

(EDTA red cells)

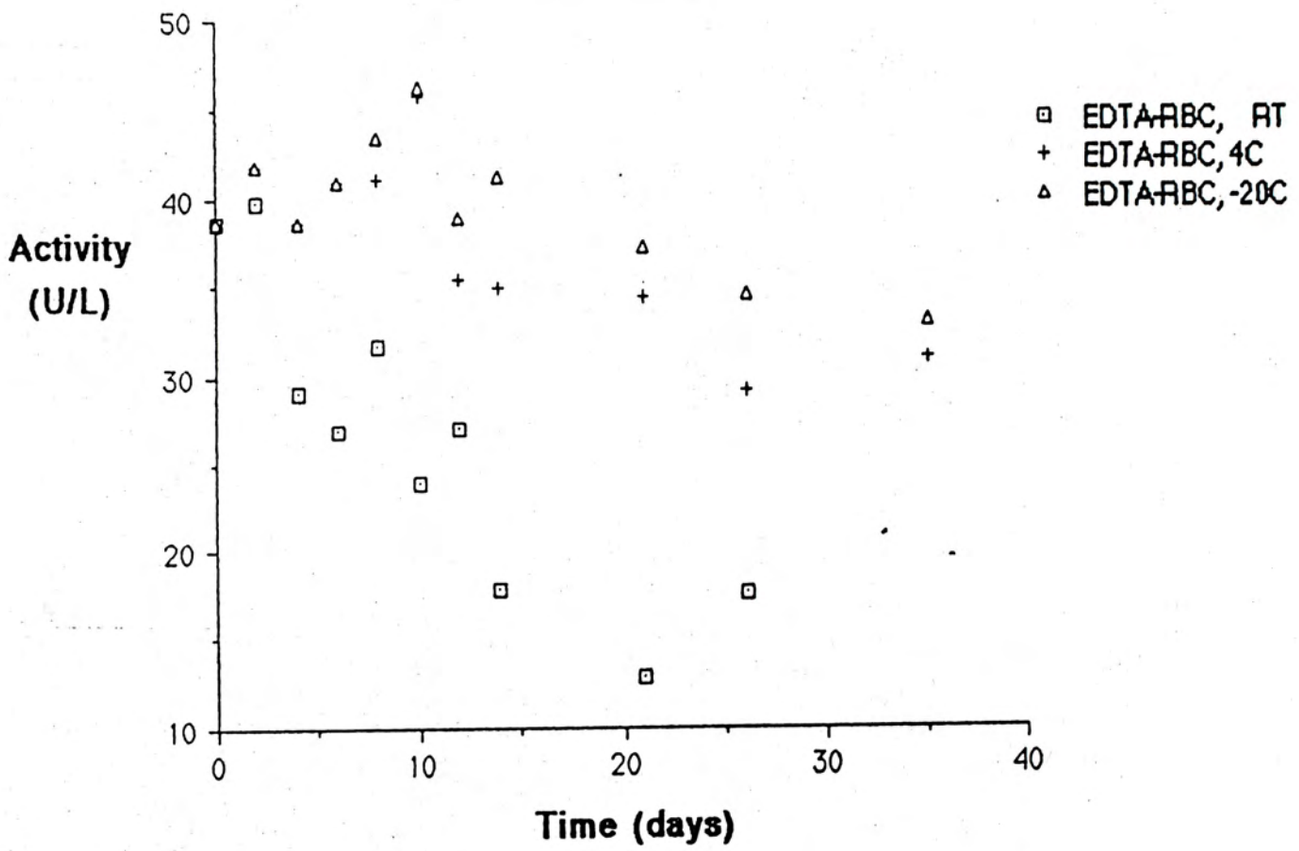


Fig. 15. **Effect of storage temperature**

(heparin red cells)

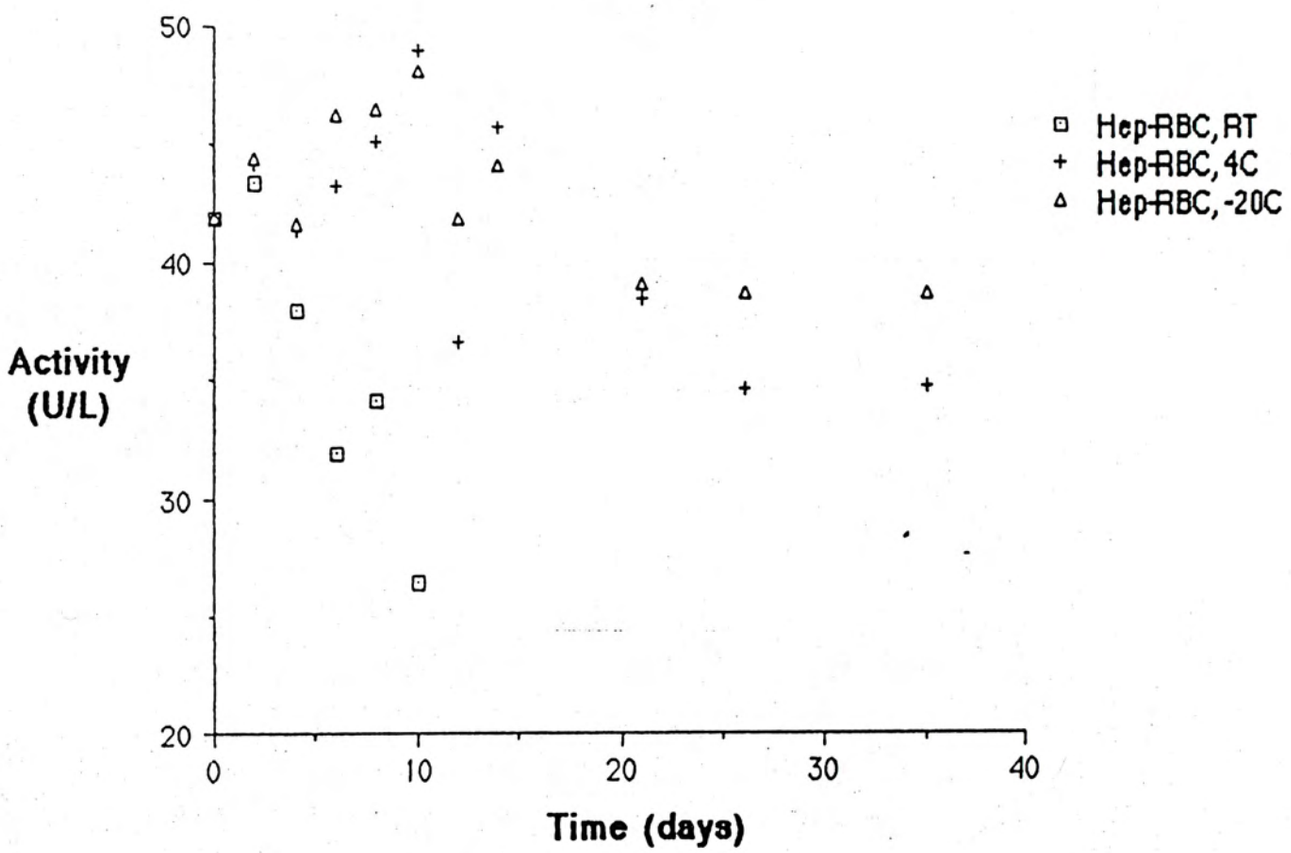


Table 7 The percentage of GSH-Px remaining after 1-week, 2-week and 5-week

Sample*	T°C +	1-week	2-week	5-week
CP	RT	58	38	19
	4	100	88	51
	-20	100	100	100
EP	RT	28	13	12
	4	94	80	30
	-20	100	100	93
HP	RT	18	12	10
	4	75	60	23
	-20	97	98	91
CH	RT	35	31	23
	4	100	100	34
	-20	100	99	88
EH	RT	38	32	M ‡
	4	95	97	51
	-20	91	92	79
HH	RT	34	28	21
	4	100	96	58
	-20	98	70	74
CR	RT	93	78	M ‡
	4	100	97	84
	-20	100	100	89
ER	RT	82	46	M ‡
	4	100	90	80
	-20	100	100	86
HR	RT	82	M ‡	M ‡
	4	100	100	83
	-20	100	100	93

* CP=citrate plasma, EP=EDTA plasma, HP=heparin plasma, CH=citrate haemolysate, EH=EDTA haemolysate, HH=heparin haemolysate, CR=citrate red cells, ER=EDTA red cells, HR=heparin red cells.

\+ T°C=Temperature, RT=room temperature.

‡ M indicates that the specimen was clearly contaminated by a growth of mould.

the enzyme in plasma quite well for 1 week, except in heparin plasma, and even 2 weeks for red cells or haemolysates. By the end of 5^{th} week, GSH-Px in plasma, haemolysate and red cell lost its activity significantly at $4^{o}C$. The data show that it is not suitable to store the samples for GSH-Px activity at room temperature.

In the preservation of GSH-Px activity in red cell, it appears that the enzyme is more stable in the form of washed red cells rather than in the haemolysate. When different anticoagulants were compared, it seemed that citrate was the best one for preservation of GSH-Px in plasma, but its effect on haemolysate or red cell was not so marked. The percentage losses of enzyme activity in EDTA and heparin samples were quite similar in magnitude.

In the stability study, there was an unexplained sudden rise in red cell and haemolysate GSH-Px activity on day 10. It may be due to a systematic error, although the controls for GSH-Px and haemoglobin did not show such a change. However, the plasma samples did not have this rise.

Biological variation In this section of project, samples were drawn from 14 volunteers on five occasions

over a period of ten weeks. It was anticipated that there would be variation in the values obtained at different times in the one subject. The simplest model to be considered was that the variation was random and the sources of variation were both analytical and biological of a random nature. Blood samples were collected in different anticoagulants : citrate, EDTA and heparin. The data of plasma and red cell GSH-Px activities in different anticoagulants and individuals are shown in (Table 8), and (Figs. 16-21). From the data of enzyme activities during the period of 10 weeks, there was a significant change in red cell GSH-Px activity with time (all three different types of red cells had F value greater than 33 and P value less than 0.0001). The red cell GSH-Px activity in the second blood sampling (1^{st} week) was generally higher. Such an increase did not occur in plasma samples. Statistically significant changes in enzyme activity in citrated and heparinised plasma samples with time were found (citrate : F=5.57; P=0.0008, heparin : F=3.86; P=0.0078) but not in EDTA plasma (F=1.73; P=0.1576). The analysis of variance showed there was a significant difference between subjects (F values for the subject effect of all six types of samples were greater than 17 and P were less than 0.0001).

Table 8 Glutathione peroxidase activity in plasma of 14 subjects

SUBJECTS		1	2	3	4	5	6	7	8	9	11 +	12 +	13 +	14	15 +
Citrate plasma	Time 0	399	312	337	369	336	317	326	365	369	386	277	536	338	305
(U/L)	1st week	403	297	238	335	310	310	333	346	344	331	260	477	325	329
	2nd week	395	309	368	389	327	308	348	395	355	325	316	503	342	329
	5th week	375	276	336	316	290	311	334	337	308	286	268	421	312	329
	10th week	374	284	330	317	309	348	332	329	337	288	295	447	384	369
EDTA plamsa	Time 0	488	364	448	430	396	389	394	432	426	421	321	601	404	367
(U/L)	1st week	464	359	416	424	411	366	388	412	412	374	307	567	402	395
	2nd week	469	371	431	465	417	384	392	456	427	375	340	581	405	402
	5th week	444	361	445	427	379	411	411	428	415	369	336	524	441	420
	10th week	457	336	404	416	371	426	396	405	418	358	346	532	464	416
Heparin plasma	Time 0	507	374	476	453	419	401	395	437	460	437	338	644	417	376
(U/L)	1st week	481	378	447	432	412	389	403	420	415	376	309	582	407	415
	2nd week	515	383	452	480	425	408	417	471	446	396	358	599	432	418
	5th week	475	372	440	427	384	424	427	429	417	376	328	550	455	437
	10th week	468	349	425	418	387	420	402	419	438	364	357	544	473	434

* Subject 10 had her maternity leave, her data were incomplete.
+ Subjects 11, 12, 13, 15 are female.

Table 8 (cont.) Glutathione peroxidase activity in red cells of 14 subjects

SUBJECTS		1	2	3	4	5	6	7	8	9	11 +	12 +	13 +	14	15 +
Citrate red cell	Time 0	24.9	30.2	33.3	27.6	41.0	28.6	28.5	57.1	32.1	29.9	29.9	31.6	32.3	36.3
(U/g Hb)	1st week	30.8	41.4	41.9	38.4	59.4	39.5	33.8	79.8	42.1	42.3	37.4	42.1	42.7	46.2
	2nd week	22.9	32.0	33.4	26.2	33.1	26.5	27.8	58.4	30.1	29.2	27.4	31.1	31.6	31.5
	5th week	22.7	32.0	37.5	26.0	43.7	29.4	30.3	59.3	31.4	27.3	27.0	29.5	31.2	36.4
	10th week	25.0	31.7	33.8	29.9	41.1	34.2	32.2	63.7	34.2	33.4	29.6	33.5	34.8	39.9
EDTA red cell	Time 0	22.2	34.5	34.8	26.5	42.9	29.6	29.9	60.1	31.9	31.3	31.2	33.7	32.8	37.2
(U/g Hb)	1st week	33.3	50.1	49.7	39.0	56.4	43.0	36.9	83.8	42.4	40.7	36.8	40.9	37.6	44.8
	2nd week	23.9	32.8	34.5	25.8	35.5	27.6	28.1	58.9	30.5	30.6	32.9	36.5	36.6	41.9
	5th week	25.5	34.6	38.0	27.1	44.3	32.4	32.5	66.4	34.3	32.8	32.7	34.9	37.2	41.1
	10th week	28.4	35.2	38.4	30.9	43.9	33.4	33.0	64.1	38.1	37.0	32.9	36.9	39.1	41.3
Heparin red cell	Time 0	22.1	32.0	34.6	27.9	43.1	30.1	31.2	66.9	32.7	32.4	32.5	34.9	35.1	40.4
(U/g Hb)	1st week	28.6	41.8	40.5	33.2	51.0	37.5	35.5	71.8	43.2	39.4	33.8	40.1	39.5	47.0
	2nd week	25.1	36.5	36.7	28.9	38.5	30.9	31.6	66.4	35.1	33.8	30.9	35.0	35.3	39.9
	5th week	25.5	36.9	37.8	30.6	45.2	34.5	32.1	66.3	30.0	36.5	32.1	35.7	37.4	38.7
	10th week	28.8	35.8	40.3	33.6	48.4	32.2	35.6	66.3	37.3	38.9	34.8	36.7	38.8	42.6

* Subject 10 had her maternity leave, her data were incomplete.
+ Subjects 11, 12, 13, 15 are female.

Fig.16. Activity at 5 points in time (Citrate/plasma)

Fig.17. Activity at 5 points in time (EDTA/plasma)

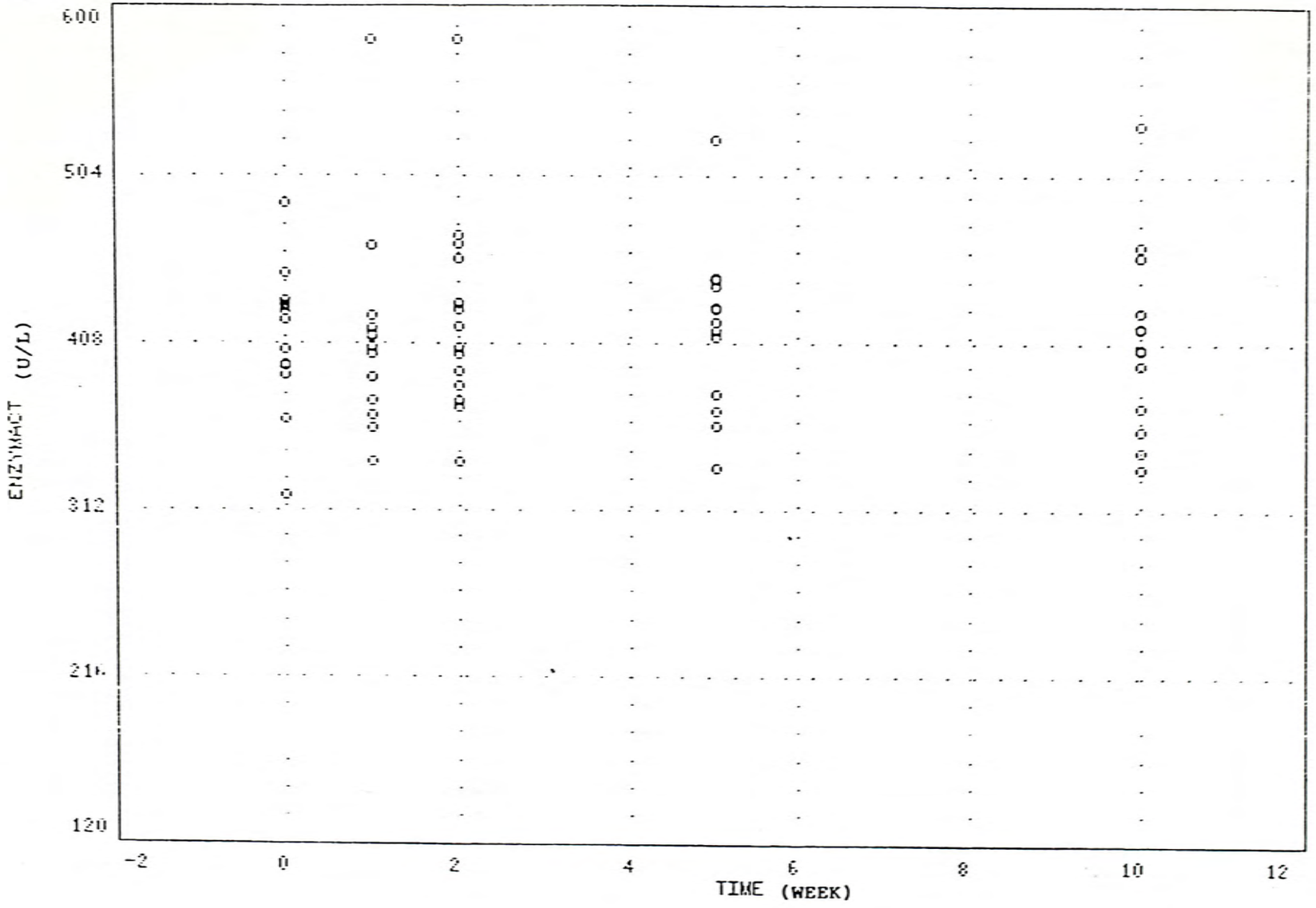


Fig.18. Activity at 5 points in time (Heparin/plasma)

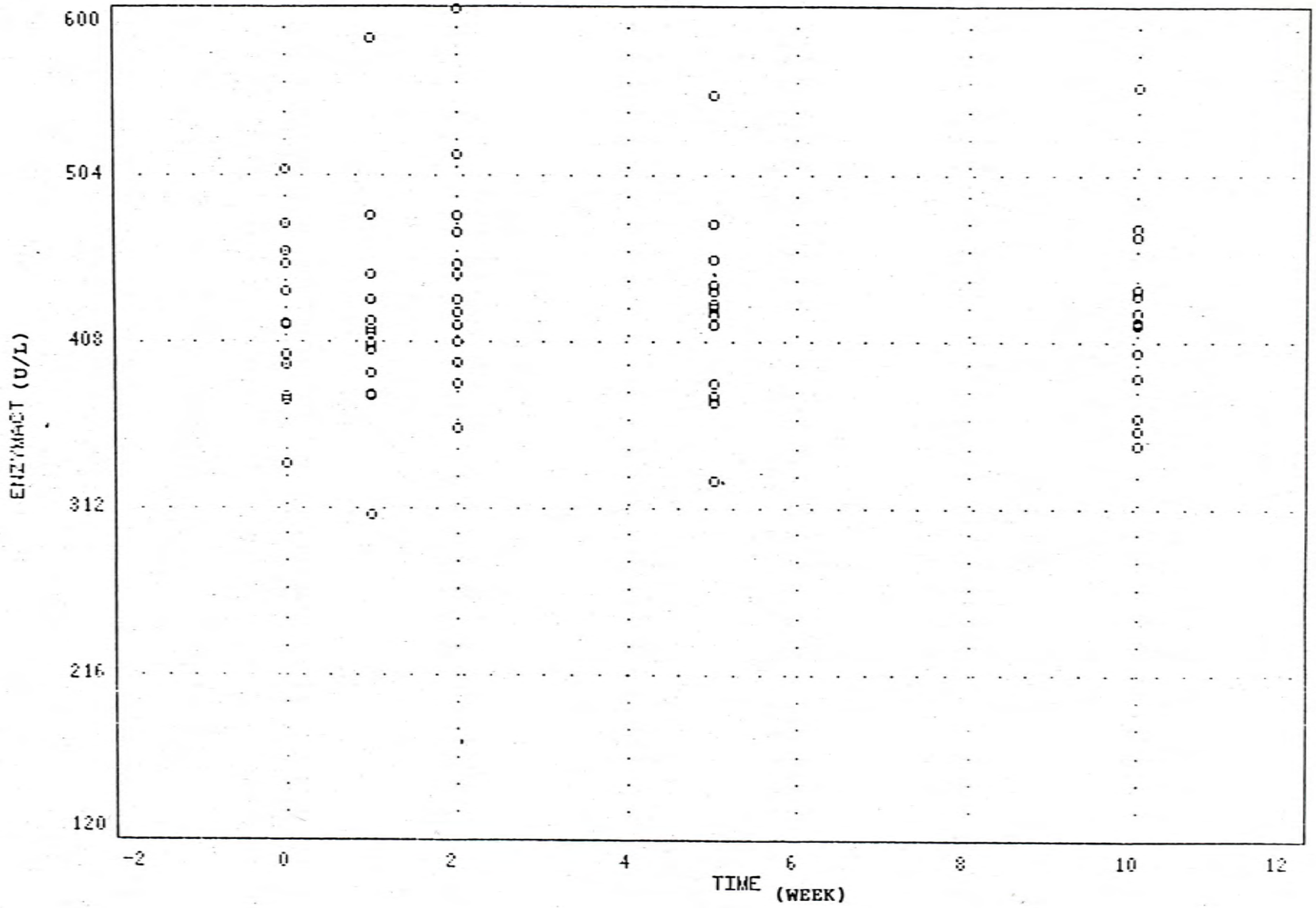
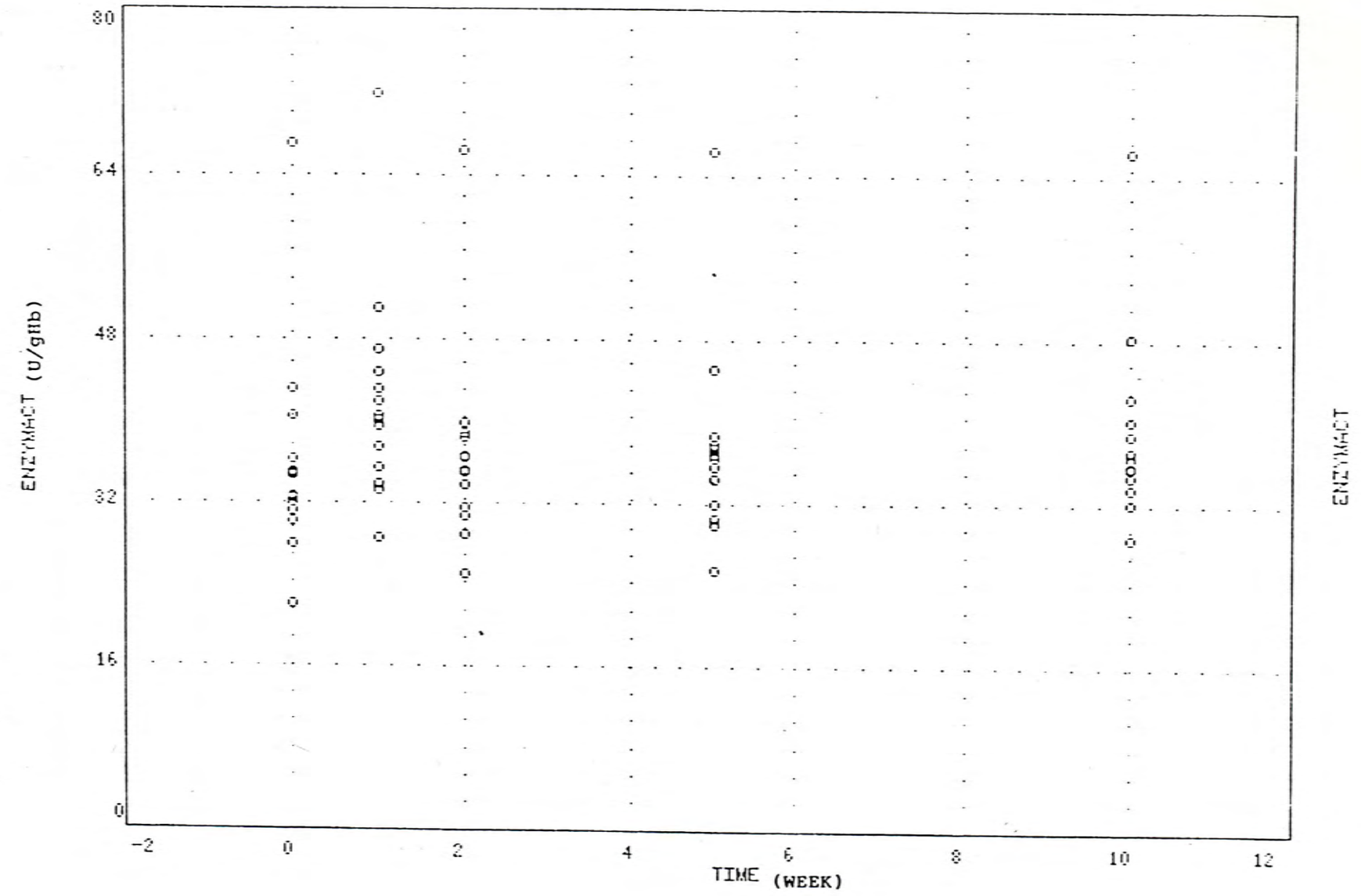


Fig.19. Activity at 5 points in time (Citrate/RBC)

Fig.20. Activity at 5 points in time (EDTA/RBC)

Fig.21. Activity at 5 points in time (Heparin/RBC)



The mean and SD of each type of specimen are shown in (Table 9). These figures could be used in establishing a reference interval for GSH-Px activity. Student's t-test showed that citrate plasma GSH-Px activity was significantly lower than heparin- and EDTA- plasma levels ($P < 0.0005$), while no statistically significant difference between heparin and EDTA plasma GSH-Px activities was detected ($0.1 < P < 0.2$). A likely explanation is that it was due to the dilutional effect on the plasma samples caused by the liquid form of citrate (1 part citrate to 9 parts blood sample). No such difference was found between washed red cell GSH-Px activities in the three anticoagulants ($P > 0.1$).

To show whether there was difference in GSH-Px activites between the sexes, the means of ten males and 4 females GSH-Px levels (Table 10) were examined by Student's t-test. In all of the 6 pairs of results, there were no statistically significant differences between male and female GSH-Px activities.

One of the major purposes in this study was to find the biological variation of GSH-Px in the health. In the determination of components of variation, for each individual, a single set of data (5 results over a period of 10 weeks) was used to calculate the SD, which

Table 9 Mean and SD of GSH-Px activity of 14 subjects

Sample*	Mean	SD
CP (U/L)	341	52.0
EP (U/L)	415	55.3
HP (U/L)	429	59.5
CR (U/gHb)	35.5	10.23
ER (U/gHb)	37.7	10.43
HR (U/gHb)	37.9	9.81

* CP = citrate plasma, EP = EDTA plasma,
HP = heparin plasma, CR = citrate red cells,
ER = EDTA red cells, HR = heparin red cells.

Table 10 Concentration of GSH-Px activity in plasma and red cells in men and women

	men (n=10) mean (SD)	women (n=4) mean (SD)	P
Plasma (U/L)			
Citrate	336(25.5)	354(84.9)	$0.1 < P < 0.2$
EDTA	414(28.9)	418(100.1)	$P > 0.5$
Heparin	428(31.7)	432(106.4)	$P > 0.5$
Red cells (U/gHb)			
Citrate	36.3(10.8)	33.6(3.30)	$0.2 < P < 0.5$
EDTA	38.2(11.2)	36.4(3.52)	$P > 0.5$
Heparin	38.3(11.4)	36.8(3.67)	$P > 0.5$

is made up of analytical imprecision (SD_A) and intraindividual biological variation (SD_{Intra}),

$$(SD)^2 = (SD_A)^2 + (SD_{Intra})^2$$

the squared SD (variance) being used because variance is the statistic which can be mathematically manipulated. From the duplicate analyses of each sample, the analytical imprecision (SD_A) could be calculated using the formula $SD_A = \sqrt{\frac{\text{sum of (differences)}^2}{2 \times \text{no. of pairs}}}$ (11)

To obtain an estimate of intraindividual biological variation, analytical variation had to be deducted from the values of SD, $(SD_{Intra})^2 = (SD)^2 - (SD_A)^2$. Irrespective of the type of anticoagulant, the values for SD, analytical imprecision (SD_A) and intraindividual variation (SD_{Intra}) of GSH-Px activities in plasma were similar in magnitude. The same was true for the different red cell preparations. The means of SD, SD_A and SD_{Intra} of 14 individuals in different types of samples are tabulated in (Table 11). It should be remarked that the SD_A of red cell GSH-Px activity may be underestimated because in the analysis of the red cell enzyme, haemolysates were assayed in duplicate and the data were used in the calculation of SD_A, but two separate haemolysates were not prepared from a red cells

Table 11 Means of SD, SD_A and SD_{Intra} of GSH-Px activity in 14 individuals in different samples

	Mean SD	Mean SD_A	Mean SD_{Intra}
Plasma (U/L)			
Citrate	25.8	5.3	25.1
EDTA	19.1	8.8	17.5
Heparin	21.8	7.1	20.4
Red cells (U/gHb)			
Citrate	5.26	2.06	4.66
EDTA	4.93	1.89	4.42
Heparin	2.91	1.10	2.62

specimen. Therefore, the analytical error associated with the preparation of the haemolysates is included in SD_{Intra}.

In a specific type of sample, from the SD_{ALL} for all single sets of data of all subjects (70 data points), which was comprised of analytical, intraindividual, and interindividual biological variation (SD_{Inter})

$$(SD_{ALL})^2 = (SD_A)^2 + (SD_{Intra})^2 + (SD_{Inter})^2$$

Therefore, the interindividual variation could be calculated when all the three parameters were known. Table 12 showed these variations. Now, the intra- and interindividual variations were available. One of the uses of these biological variation figures was the assessment of population based reference values. A ratio could be calculated from : $\sqrt{\frac{(SD_{Intra})^2}{(SD_{Inter})^2}}$ (11)

If intraindividual biological variation is greater than interindividual variation, conventional population based reference values will be of value, particularly when this ratio is greater than 1.4. However, reference values will be of limited use and may give misleading impressions especially when the ratio is less than 0.6. As shown in (Table 13), all the ratios of different types of plasma and red cell GSH-Px activity are less

Table 12 SD_{ALL}, SD_A, SD_{Intra} and SD_{Inter} of GSH-Px activity in 14 subjects in different samples

	SD_{ALL}	SD_A	SD_{Intra}	SD_{Inter}
Plasma (U/L)				
Citrate	52.0	5.3	25.1	45.2
EDTA	55.3	8.8	17.5	51.7
Heparin	59.5	7.1	20.4	55.4
Red cells (U/gHb)				
Citrate	10.23	2.06	4.66	8.87
EDTA	10.43	1.89	4.42	9.26
Heparin	9.81	1.10	2.62	9.39

Table 13 The ratio $\sqrt{\frac{(SD_{Intra})^2}{(SD_{Inter})^2}}$ of GSH-Px activity

	SD_{Intra}	SD_{Inter}	Ratio
Plasma (U/L)			
Citrate	25.1	45.2	0.56
EDTA	17.5	51.7	0.34
Heparin	20.4	55.4	0.37
Red cells (U/gHb)			
Citrate	4.66	8.87	0.53
EDTA	4.42	9.26	0.48
Heparin	2.62	9.39	0.28

than 0.6 which implies that the reference intervals of GSH-Px in plasma and red cells would be of little use as there is a high degree of individuality. (Figs. 22-23)

DISCUSSION

The attempt to prepare platelet lysates with the use of saponin ended in failure. It may have been due to problems in the resuspension and lysis of the platelet pellet by saponin. In other researches, sonication or digitonin has been used in the lysis of platelets. If the instrument for sonication, or the chemical digitonin which has also been used were available, the platelet assay might have been feasible and have made this project more informative.

In the determination of GSH-Px activity in haemolysates, several additional chemicals or reagents were used. They were Drabkin's reagent which was incorporated with cyanide, and azide. It was believed that if Drabkin's reagent was not added in the assay system, H_2O_2 would convert oxyhaemoglobin to methaemoglobin, allowing NADPH conversion by a direct nonenzymatic reaction or through the mediation of methaemoglobin reductase. The oxyhaemoglobin in

Fig. 22. Intraindividual variation in enzyme activity
(citrate plasma: bars represent mean ± 2SD)

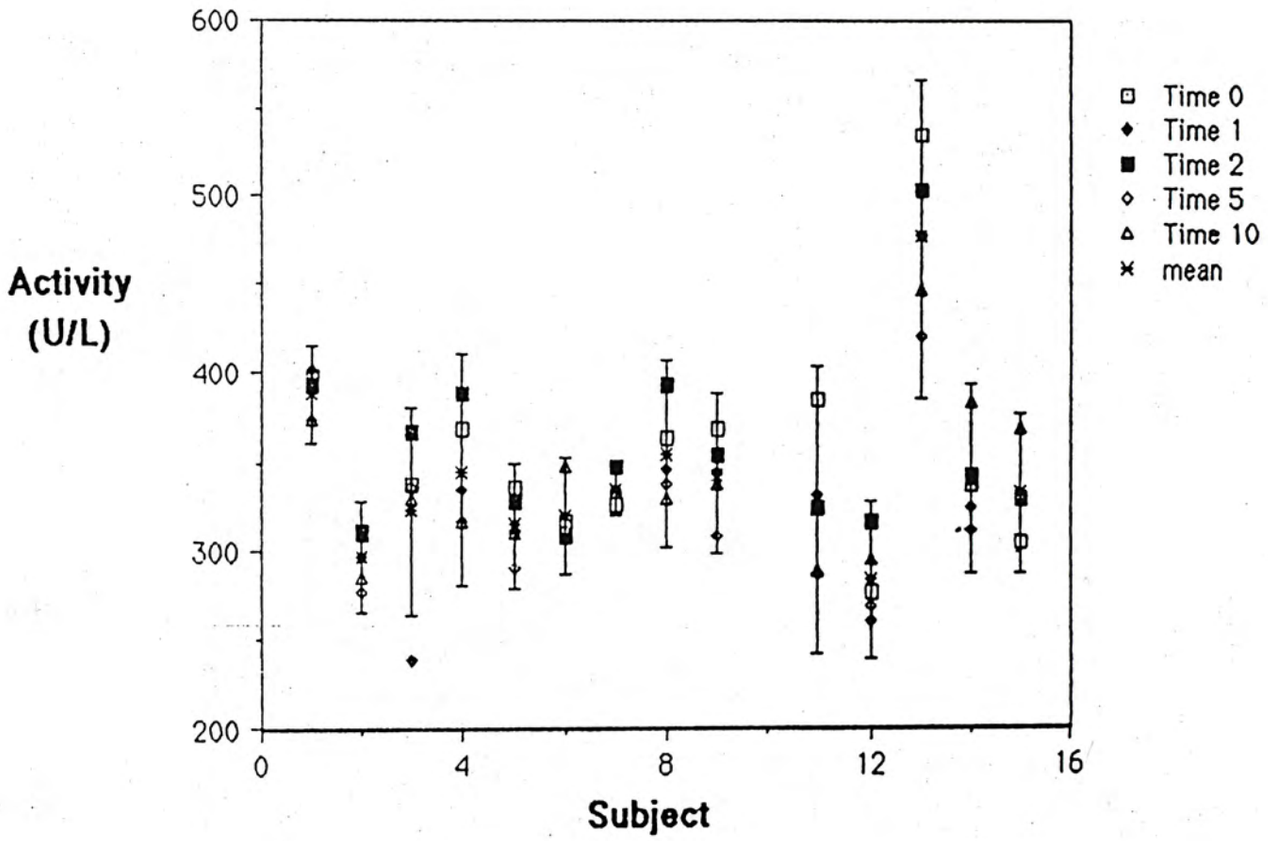
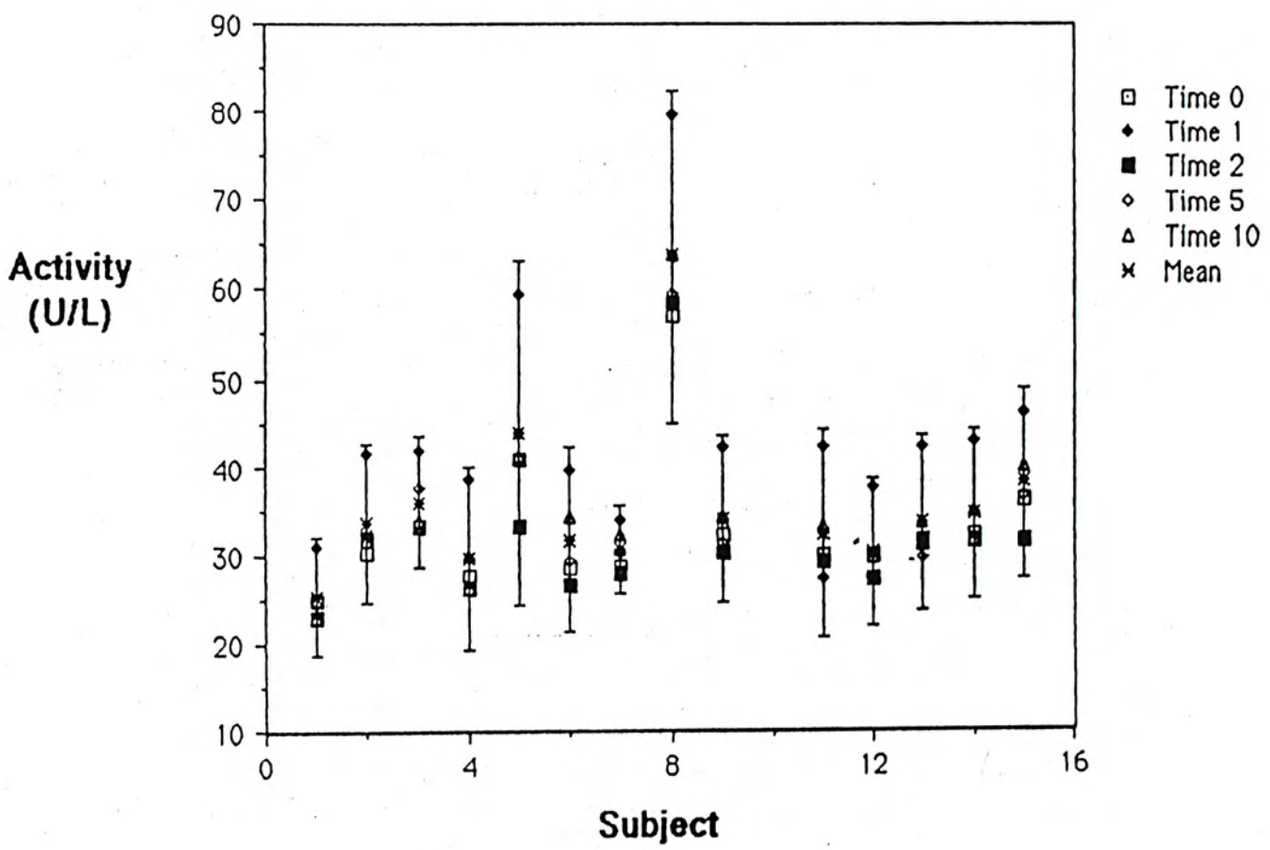


Fig. 23. Intraindividual variation in enzyme activity
(citrate/RBC: bars represent means ± 2SD)



haemolysate could be converted to the stable cyanmethaemoglobin by Drabkin's reagent and therefore the interference with the strict measurement of GSH-Px activity was eliminated. Cyanide or azide acted as a catalase inhibitor to prevent decomposition of H_2O_2 by red cell catalase in the reaction system. The cyanide contributed by the Drabkin's solution was found insufficient in concentration to inhibit totally the endogenous catalase. Addition of azide was necessary to reach the catalase-blocking level. Since the activity of GSH-Px appears to be dependent upon active sulfhydryl groups and was unaffected by low concentrations of azide, cyanide or ferricyanide, the GSH-Px activity in plasma sample could also be measured with reagent incorporated with these components (9). The problem of non-linearity in the haemolysate assay using reagents suggested by McMaster et al (5) was due to the insufficiency of azide in the reaction system and failure to suppress the endogenous catalase. After increasing the azide concentration a thousand fold, a nearly linear reaction profile could be obtained.

No report could be found giving a detailed discussion on the storage stability of GSH-Px at different temperatures and in different anticoagulants. Information in this area sought in this study. Like

other enzymes, GSH-Px was best preserved at low temperature (-20°C), and was unstable at room temperature. The enzyme activity in plasma at -20°C in general did not drop significantly at least for 4-5 weeks. In haemolysate and red cell, GSH-Px could be kept for at least 3 weeks at -20°C. It was interesting that at 4°C the enzyme in haemolysate and red cell was quite stable for about 3 weeks, but not in plasma. Among the three anticoagulants, it was found that citrate was apparently better than the other two in keeping GSH-Px activity especially in plasma sample, because the percentage drops in enzyme activity of samples stored at different temperatures were comparatively smaller. However, the activity of GSH-Px in citrate plasma was significantly lower, which may have been due to the dilution effect contributed by the liquid form of citrate. Compared with the red cell, the enzyme was not as stable in haemolysates. As a result, and in order to preserve intracellular GSH-Px in blood cell, it is considered best to keep the washed red cells at -20°C.

The analysis of variance showed that except in EDTA plasma, there were statistically significant changes of GSH-Px activity in both red cells and plasma with time. The changes in red cell samples were much greater than

the plasma samples. This might have been due to the generally higher levels in red cell enzyme activity in the second blood sampling (week 1). We cannot exclude some other factors such as environmental and seasonal changes as the causes for this increase. It appears more likely that the analysis of variance has detected a systematic analytical variation that was not apparent from the results of quality control specimens assayed at the time. However, the significant changes in plasma enzyme activity might indicate that GSH-Px activity does fluctuate with time due to environmental effects and etc.

Changes in numerical laboratory results can occur with time following acute disease processes, physiological or pharmacological stimulation or suppression. Moreover, marked changes happen over a lifetime, especially during the neonatal period and childhood and in the elderly. Some body constituents show regular rhythmic changes which may be daily, monthly or seasonal in nature. It is important to know these changes otherwise specimens may not be collected at appropriate times, wrong reference values may be used and correct interpretation of test results cannot be achieved. Most analytes do not exhibit rhythmical variation but rather show random variation which is due

to two sources : analytical imprecision and biological variation. The biological variation around the homeostatic setting-point in an individual is called the intraindividual variation and the differences between the setting-points of individuals is called the interindividual variation. Schneider found : "No matter how finely we restrict a group of healthy persons by age, sex and other criteria, and no matter how narrowly we restrict our observations in the same individual with respect to time of day, relation to meals and physiologic conditions, in general we are left with unexplained variability from time to time and from person to person." (12). Analytical variation is mainly contributed by the inconsistency in collecting the specimen, processing of the specimen and subsequent analysis. The intra-individual biological variation may be influenced by several factors such as diurnal effect, hormones, posture, diet, exercise and seasonal changes. Age, sex, race, environment, genetics, stress and medication are believed to be important factors that influence interindividual variation.

Biological variation data can be used in patient care to assess the significance of changes in serial results obtained on an individual, to determine the usefulness or otherwise of conventional population based

reference values and to set desirable standards of analytical performance. The biological variation of GSH-Px in young healthy subjects was investigated in this study and the data applied to the various uses mentioned above.

In the study, the 14 healthy volunteers were not constrained in any aspect of their lifestyles and were not on any drugs. Blood samples were taken at the same time of day to eliminate variations caused by circadian rhythms. However, they were not requested to fast before blood sampling and their physical activity, and stress levels were not known. If these parameters were controlled, the pre-analytical variation would have been further minimized.

One of the uses of biological variation data is to establish whether two test results from a single patient are statistically significantly different. It can be based upon the SD of the test which includes analytical random variation and biological variation, where $SD = \sqrt{(SD_{analytical})^2 + (SD_{biological})^2}$. If the value of difference between two test results is about 2.8 times of SD, it can be claimed that it is 95% certain that two results differ. Ideally, clinical laboratories can provide data about biological variation and

analytical variation achieved in their own laboratory and give this information to the clinicians so that better interpretation of numerical data can be made with good knowledge of both relevant analytical and biological variation (11).

The ratio $\left[(SD_{Intra})^2/(SD_{Inter})^2 \right]^{\frac{1}{2}}$ provides an index for judging whether the conventional population-based reference range is likely to detect a statistically significant change within a given individual. Briefly, when this ratio is less than 0.6, the reference range will be less sensitive to change than the individual's own record of test measurements during the past. The 95% reference range derived from a large group may include a greater-than-95% proportion of the individual's distribution of values over a period of time. It will not be of significant help to interpretation because individuals may have marked changes with constituent levels significantly different from their own usual values but all these results may still lie within the usual reference interval. On the other hand, if intraindividual biological variation is greater than intraindividual variation, and particularly when the ratio exceeds 1.4, conventional population based reference values will be of value. Generally, the 95% reference range includes less than 95% of the

individual's usual distribution of values. However, for subjects in whom the concentration of a serum constituent is kept within a relatively narrower values, the population-based range remains less sensitive to charges (13). Using the ratio of average within-subject variance to the variance among subjects as a guide, both plasma and red cell GSH-Px activities showed great individuality, the population-based reference range for GSH-Px was found to be either insensitive or irrelevant.

It is not surprising to have reference range of common clinical tests which are found to be of limited use. In the study by Harris E.K. et al (14), the usefulness of reference ranges of laboratory tests (renal function test, liver function test, glucose, calcium phosphate and cholesterol) were investigated. It was found that even including analytical variation, 12 of the 18 ratio $\sqrt{(SD_{Intra})^2/(SD_{Inter})^2}$ values were less than unity. In five of the six remaining tests (sodium, potassium, carbon dioxide, calcium, and AST in women), the normal range was of marginal validity in terms of intraindividual variation ($1.2 < r < 1.4$). Therefore, the population-based range would be regarded as insensitive to individual changes. The implication is clear that routine assessment of individual laboratory tests by reference to conventional,

population-based normal ranges should no longer remain an accepted, automatic practice. In at least some tests, individual normal ranges may be necessary for reliable interpretation of results.

It is generally accepted that the goals for imprecision should be based upon biological variation. Low analytical imprecision will be required if biological variation around the homeostatic setting-point is small. The tolerable analytical variability should be equal to or less than one half the relevant biological variation. (11)

$$SD_{analytical} \leq \tfrac{1}{2} SD_{Intraindividual}$$

Referring to table 12, it may be claimed that the analytical goals for the performance characteristics of GSH-Px assay are achieved because the SD_A values were much lower than half of SD_{Intra} values.

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